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Expression of *c-fos* in the Rat Brainstem After Exposure to Hypoxia and to Normoxic and Hyperoxic Hypercapnia

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ABSTRACT

In this study, Fos immunohistochemistry was used to map brainstem neuronal pathways activated during hypercapnia and hypoxia. Conscious rats were exposed to six different gas mixtures: (a) air ; (b) 8% CO_2 in air; (c) 10% CO_2 in air; (d) 15% CO_2 in air; (e) 15% CO_2 + 60% O_2, balance N_2; (f) 9% O_2, balance N_2. Double-staining was performed to show the presence of tyrosine hydroxylase. Hypercapnia, in a dose-dependent way, caused Fos expression in the following areas: caudal nucleus tractus solitarius (NTS), with few labeled A2 noradrenergic neurons; noradrenergic A1 cells and noncatecholaminergic neurons in the caudal ventrolateral medulla; raphe magnus and gigantocellular nucleus pars α (GiA); many noncatecholaminergic (and relatively few C1) neurons in the lateral paragigantocellular nucleus (PGCl), and in the retrotrapezoid nucleus (RTN); locus coeruleus (LC), external lateral parabrachial and Kölliker-Fuse nuclei, and A5 noradrenergic neurons at pontine level; and in caudal mesencephalon, the ventrolateral column of the periaqueductal gray (vlPAG). In most of these nuclei, hypoxia also induced Fos expression, albeit generally less than after hypercapnia. However, hypoxia did not cause labeling in RTN, juxtafacial PGCl, GiA, LC, or vlPAG. After normoxic hypercapnia, more labeled cells were present in NTS and PGCl than after hyperoxic hypercapnia. Part of the observed labeling could be caused by stress- or cardiovascular-related sequelae of hypoxia and hypercapnia. Possible implications for the neural control of breathing are also discussed, particularly with regard to the finding that several nuclei, not belonging to the classical brainstem respiratory centres, contained labeled cells. J. Comp. Neurol. 388:169–190, 1997. © 1997 Wiley-Liss, Inc.

Indexing terms: peripheral and central chemoreceptors; central respiratory neural network; medulla oblongata; pons; mesencephalon

In the control system regulating pulmonary ventilation, the tensions of oxygen and carbon dioxide (Pa_{CO_2} and Pa_{O_2}) are considered as controlled variables. Disturbances in Pa_{O_2} or Pa_{CO_2} are measured by the peripheral and central chemoreceptors, located in the carotid bodies and brainstem, respectively. In the brainstem, afferent input from both categories of chemoreceptors is processed and integrated with other inputs to yield a final command to the respiratory motoneurons.

Numerous studies have been devoted to the location and characterization of the elements of the brainstem respiratory neural network. Within the medulla oblongata, respiratory neurons are mainly concentrated within the nucleus tractus solitarius (NTS) and within a ventrolaterally located cell-column, extending approximately from the highest cervical level to the Bötzinger complex (see extensive review by Feldman, 1986). Pontine respiratory neurons are mainly located in the medial parabrachial and Kölliker-Fuse nuclei (Feldman, 1986). Hypoxia and hypercapnia result in altered activity of respiratory neurons, and an important issue in respiratory physiology relates to the question of which neuro-anatomical pathways and neurotransmitters are responsible for the processing of afferent

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input from both the peripheral and central chemoreceptors.

In the rat, chemo-afferent traffic from the carotid bodies reaches the brainstem, with glossopharyngeal nerve fibres terminating preferentially in the caudal NTS (Housley et al., 1987; Finley and Katz, 1992; Chitravanashi and Sapru, 1995). Existing pathways from the NTS to respiratory regions in the rostroventrolateral medulla (RVLM) and pons (e.g., see Ross et al., 1985; Ter Horst and Streefland, 1994) may be involved in further processing of information from the peripheral chemoreceptors. Much less is known about the fate of the output from the central chemoreceptors. This situation is complicated by the fact that, despite an agreement on the existence of superficial respiratory chemosensitive regions within the caudal and rostral lateral ventral medulla (see review by Schlaefke, 1981), as yet an exclusive localization and identification of specific CO_2 sensors in these areas have proven to be elusive. The NTS, raphe nuclei, locus coeruleus and even diencephalic structures may also contain central chemoreceptors (Dean et al., 1990; Dillon and Waldrop, 1992; Coates et al., 1993; Richerson, 1995; Bernard et al., 1996).

An attractive and widely accepted tool to map multisynaptic neuronal pathways is Fos immunohistochemistry (Sagar et al., 1988; Dragunow and Faull, 1989; Bullit, 1990). The expression of the proto-oncogene *c-fos* is considered to be a marker of activation of individual neurons after synaptic activation and Ca^{2+} influx through voltage-sensitive Ca^{2+} channels (Morgan and Curran, 1986, 1989, 1991; Sheng and Greenberg, 1990). The protein product of *c-fos*, the nuclear protein Fos, is rapidly and transiently induced, remains in the cell nucleus at elevated concentrations for hours, and can be detected immunohistochemically with antibodies. In several studies, Fos immunohistochemistry was used to map neuronal pathways involved in the physiological responses to hypoxia and hypercapnia. Erickson and Millhorn (1994) and Larnicol et al. (1994) studied the expression of *c-fos* in the brainstem during hypoxia in awake or anesthetized rats and awake cats, respectively. The effects of hypercapnia were studied in the awake and anesthetized cat (Larnicol et al., 1994; Teppema et al., 1994, respectively) and in anesthetized, as well as awake rats (Sato et al., 1992; Miura et al., 1994; Teppema et al., 1995). The hypercapnic studies, however, did not yield a clear picture because the reported data and interpretations were equivocal. This is probably due to variations in technical and experimental conditions such as handling, degree of accommodation, stimulus intensity and duration, and the use of different anesthetic regimens. In the chloralose-urethane anesthetized cat, inhalation of 10% CO_2 for 60 minutes results in *c-fos* expression in the retrotrapezoid nucleus (RTN, Teppema et al., 1994), whereas in awake animals, several intermittent exposures to 5% CO_2, each with a duration of about 30 minutes, does not (Larnicol et al., 1994). In the awake rat, a CO_2 concentration as high as 13% was necessary to induce *c-fos* in the medullary chemosensitive areas and in the NTS (Sato et al., 1992). However, awake rats exposed to 15% CO_2 for 2 hours show a different pattern of labeling in the

Abbreviations

A1	noradrenergic cell group in caudal ventral medulla	NTS	nucleus of the solitary tract
AMBd	nucleus ambiguus, dorsal division	PAG	periaqueductal gray
AMBv	nucleus ambiguus, ventral division	$PaCO_2$	partial pressure of CO_2 in arterial blood
AP	area postrema	PaO_2	partial pressure of O_2 in arterial blood
AQ	cerebral aqueduct	PB	parabrachial nucleus
C1	adrenergic cell group in rostral ventral medulla	PBle	parabrachial nucleus, external lateral part
cc	central canal	PBm	parabrachial nucleus, medial part
cic	commissure of the inferior colliculus	PBmm	parabrachial nucleus, medial medial part
CSN	carotid sinus nerve	PCG	pontine central gray
cst	corticospinal tract	PG	pontine gray
CU	cuneate nucleus	PGCl	paragigantocellular nucleus, lateral part
CUN	cuneiform nucleus	POR	periolivary nucleus
CVLM	caudal ventral lateral medulla	PRNr	pontine reticular nucleus, rostral part
CVRG	caudal ventral respiratory group	PSV	principal sensory nucleus of the trigeminal
DCO	dorsal cochlear nucleus	py	pyramidal tract
DMX	dorsal motor nucleus of vagus nerve	RM	nucleus raphe magnus
DR	dorsal nucleus raphe	RO	nucleus raphe obscurus
Fos-LI	Fos-like immunoreactivity	RPA	nucleus raphe pallidus
GiA	gigantocellular nucleus pars α	RTN	retrotrapezoid nucleus
GR	gracile nucleus	RVLM	rostral ventral lateral medulla
GRN	gigantocellular reticular nucleus	RVMM	rostral ventral medial medulla
gVIIn	genu of the facial nerve	scp	superior cerebellar peduncle
IC	inferior colliculus	SPV	spinal nucleus of the trigeminal
IO	inferior olivary nucleus	SPIV	spinal vestibular nucleus
IVLM	intermediate ventral lateral medulla	SPVOvl	spinal nucleus of the trigeminal, oral part, ventrolateral part
KF	Kölliker-Fuse nucleus		
LAV	lateral vestibular nucleus	TH	tyrosine hydroxylase
LC	locus coeruleus	TRN	tegmental reticular nucleus, pontine gray
LDT	laterodorsal tegmental nucleus	ts	solitary tract
LRN	lateral reticular nucleus	V	motor nucleus of the trigeminal nerve
le	parabrachial nucleus, external lateral part	V4	fourth ventricle
m	parabrachial nucleus, medial part	VII	facial nucleus
MARN	magnocellular reticular nucleus	VIIIn	vestibulocochlear nerve
MDRNv	medullary reticular nucleus, ventral part	VIIn	facial nerve
ml	medial lemniscus	vlPAG	ventrolateral column of periaqueductal gray
mtv	mesencephalic tract of the trigeminus	Vn	trigeminal nerve
MV	medial vestibular nucleus	vms	ventral medullary surface
NI	nucleus incertus	XII	hypoglossal nucleus
NLL	nucleus of the lateral lemniscus		

ventral medulla, with Fos-positive cells in deeper layers as well as in the RTN (Teppema et al., 1995).

The aim of the present study was to systematically compare the expression of *c-fos* in the brainstem of awake rats that were subjected to different inspiratory loads. First, to investigate the influence of the intensity of a hypercapnic stimulus, four groups of animals were exposed to inhalation of, respectively, 0, 8, 10, and 15% CO_2 in air for 2 hours. Second, to investigate a possible contribution of the peripheral chemoreceptors to *c-fos* expression, a fifth group of animals was exposed to hyperoxic hypercapnia (60% O_2 and 15% CO_2, balance N_2). Third, to compare the effects of hypercapnia with those of hypoxia, a number of animals were challenged with inhalation of 9% O_2, balance N_2. Double-staining directed to immunohistochemical demonstration of tyrosine hydroxylase (TH) enabled us to compare our results with those obtained after different hemodynamic paradigms when catecholaminergic neurons in dorsal and ventral medulla are activated (e.g., Chan and Sawchenko, 1994; Li and Dampney, 1994; Murphy et al., 1994). Because in Fos studies it is important to minimize nonspecific stress (see Dragunow and Faull, 1989), we used only frequently handled animals that were well adapted to the environmental experimental room conditions.

MATERIALS AND METHODS

Experimental protocol

The experiments were performed in adult rats (230–250 g), caged in groups of two or three animals, with food and water freely available, and at a room temperature (RT) of 23–25°C. To adapt the animals to the environmental conditions, they were kept in the experimental room for at least 10 days during which they were handled daily. To minimize nonspecific stress during the final study, the animals were daily exposed to conditions identical to those during the actual experiments, except that they breathed normal air. To minimize novelty and behavioral stress, the animals were allowed to remain in their own cage during the final experiment and were not confronted with unacquainted congeners.

The inhalation experiments were performed between 11:00 AM and 15:00 PM. The cages were provided with air-tight covers with holes, designed to use the cages as flow chambers (flow 5 liters/minute). Awake animals were exposed for 2 hours to one of the following gas mixtures (RT 23–25°C): (1) air (n = 9); (2) 8% CO_2 in air (n = 4); (3) 10% CO_2 in air (n = 5); (4) 15% CO_2 in air (n = 5); (5) 15% CO_2 + 60% O_2 balance N_2 (n = 5); (6) 9% O_2 balance N_2 (n = 7). Oxygen and carbon dioxide tensions in the chamber were measured with a fast responding zirconium oxide cell (Jaeger O_2-test, Würzburg, Germany), and an infrared analyzer (Gould Godard, Mk2 Capnograph, Bilthoven, The Netherlands). The gas concentrations were regulated with mass flow controllers (type AFC 260, Advanced Semiconductor Materials, De Bilt, The Netherlands).

Immunohistochemistry

At the end of the experiments, the animals were immediately anesthetized with a mixture of α-chloralose and urethane (70 and 700 mg/kg, respectively, i.p.). Thereafter, they were briefly perfused transcardially with heparinized saline at 4°C, followed by 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS) (pH 7.3). The brain was removed and, after postfixation of approximately 4 hours, stored overnight at 4°C in 20% saccharose in 0.1 M PBS. The next day, coronal sections (60 µm) were made from the brainstem (medulla oblongata to caudal mesencephalon) with a freezing microtome. The sections were collected in two parallel series in 0.1 M PBS in such a way as to subject one series to immunostaining for Fos, and the other to staining for both Fos and TH. One section per 600 µm was processed for cresyl violet staining. After preincubation for 1 hour with 0.1 M PBS containing 0.5% Triton X-100 and 1% bovine serum albumin (solution A), the sections were incubated (free-floating immersion) for 3 × 24 hours at 4°C with solution A to which a primary Fos antibody, raised in sheep (CRB OA-11-823; 1:10,000), was added. Thereafter, the sections were thoroughly rinsed (3 x 20 minutes each) in 0.1 M PBS and incubated for 1 hour at RT with biotinylated donkey-anti-sheep Immunoglobulin (Jackson Laboratories, Westgrove, PA; 1:800) in solution A. Then, after three washes (3 × 20 minutes), they were incubated with an avidin-biotin-peroxidase complex (ABC, 1:800; Vector Laboratories, Burlingame, CA) for 2 hours. Visualisation of the reaction product was achieved by incubation (10 minutes at RT) with a chromogen solution consisting of 0.02% 3,3'-diaminobenzidine tetrachloride (DAB) and 0.03% Ni-ammoniumsulphate in 0.05 M Tris buffer (pH 7.6) to which hydrogen peroxide (10 µl of a 30% solution per 100 ml of reaction solution) was added. Immunohistochemical controls on sections obtained from three animals subjected to inhalation of 15% CO_2 were performed by omitting the primary Fos antibody. No staining was observed in these sections. Processing of sections from test-animals was always performed in parallel with that from at least one control animal.

For double staining, sections from 15 animals were subsequently incubated overnight at RT with solution A containing antibodies against TH (1:20,000; Incstar Corp., Stillwater, MN), raised in mouse. Then, after thorough rinsing in 0.1M PBS (3 × 20 minutes), they were incubated with a biotinylated donkey anti-mouse immunoglobulin (1:800; Jackson Laboratories) in solution A for 1 hour. Finally, after another thorough rinsing (3 × 20 minutes), incubation with ABC (1:800; Vector Laboratories) in solution A was performed. Then, after three rinses in 0.1 M PBS, pH 7.6, visualization was accomplished by incubation (10 minutes at RT) with Tris buffer containing 0.02% DAB (pH 7.6) to which hydrogen peroxide (10 µl of a 30% solution per 100 ml of reaction solution) was added. After completion of the immunocytochemical procedures, the sections were dehydrated, cleared in xylene, and coverslipped.

Quantitation

Sections were analyzed by using a Zeiss light microscope. The distribution of Fos-positive nuclei was plotted onto drawings which were derived from the atlas of Swanson (1992), each of which showed a number of clearly recognizable anatomical structures. In all rats, counts of Fos immunoreactive nuclei were made at seven identical, or nearly identical, rostrocaudal levels, corresponding as closely as possible with seven reference levels taken from Swanson (1992). These seven rostrocaudal levels were the following: Level 1 (Fig. 1, row 1, approximately -14.36 from bregma), dorsally, this includes the caudal NTS and ventrally the lateral reticular nucleus (LRN), the ventral part

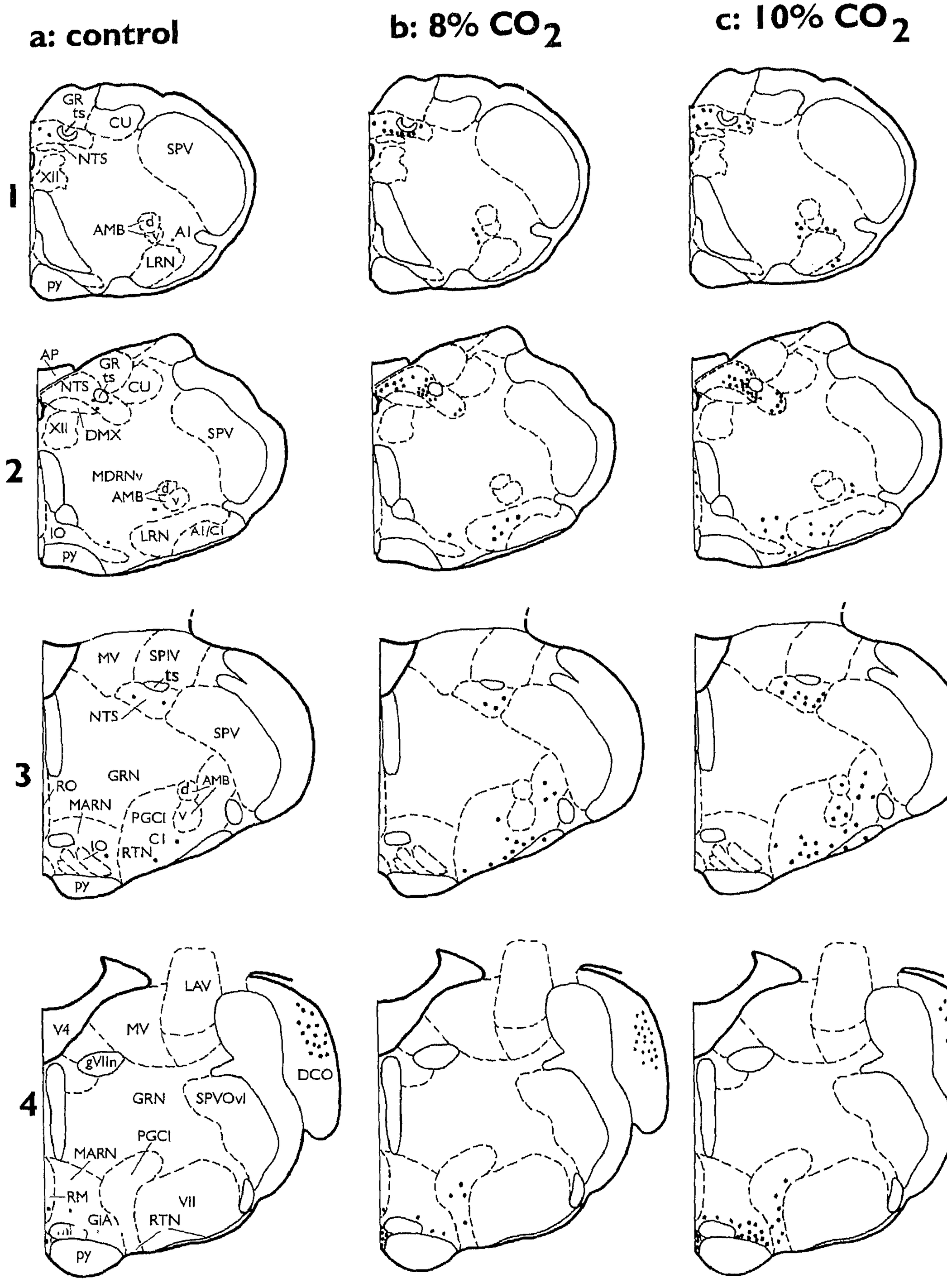
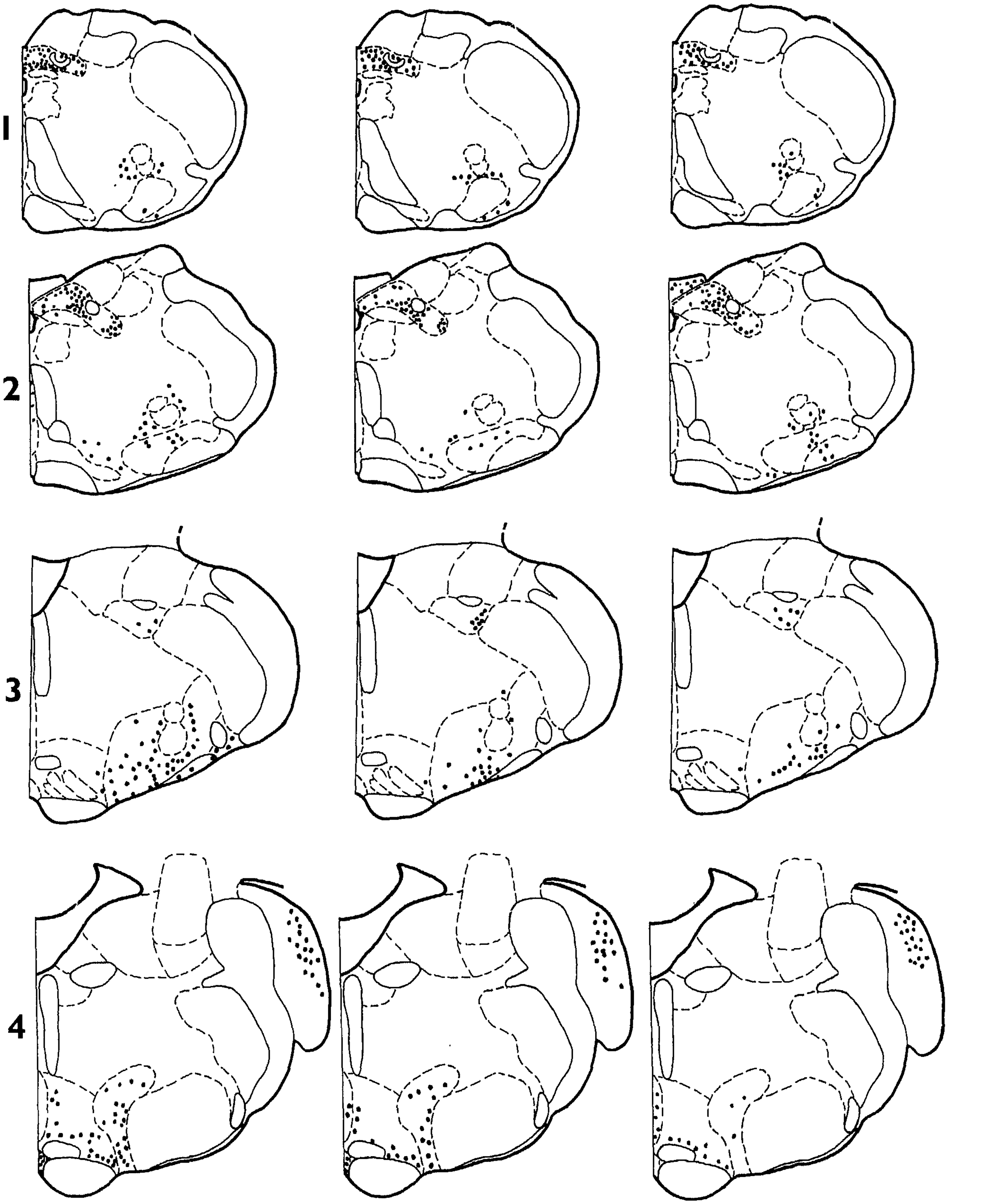


Fig. 1. Fos expression in the brainstem of the rat at seven rostrocaudal levels (rows 1–7) after six different inspiratory loads (columns a–f). Black dots represent Fos-positive neurons (approximately one dot for each two cells for levels 1–4 and one dot per four cells for levels 5–7). Sections were redrawn after that of Swanson (1992). For abbreviations, see list.

d: 15% CO_2 e: 15% CO_2 + 60% O_2 f: 9 % O_2



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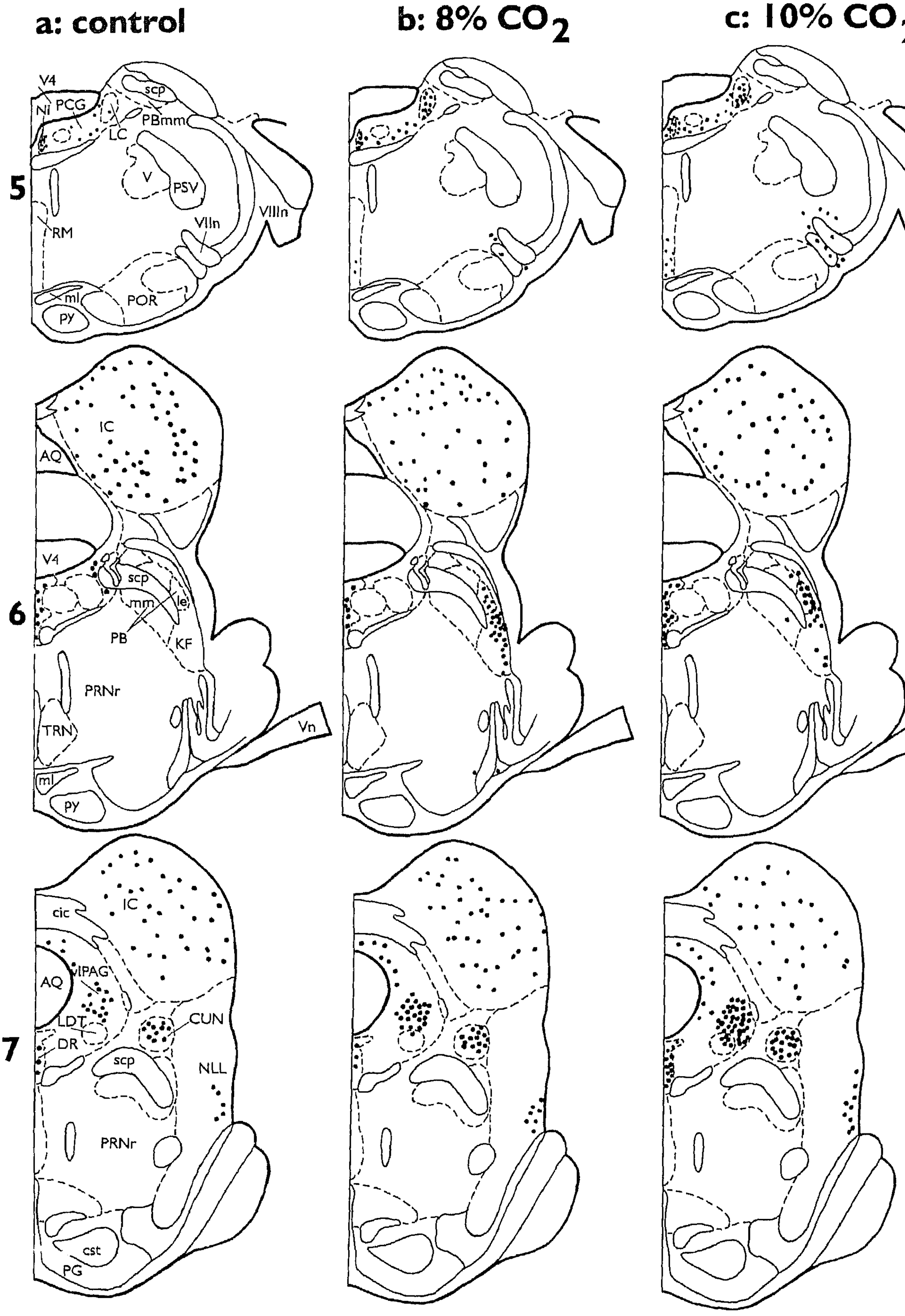

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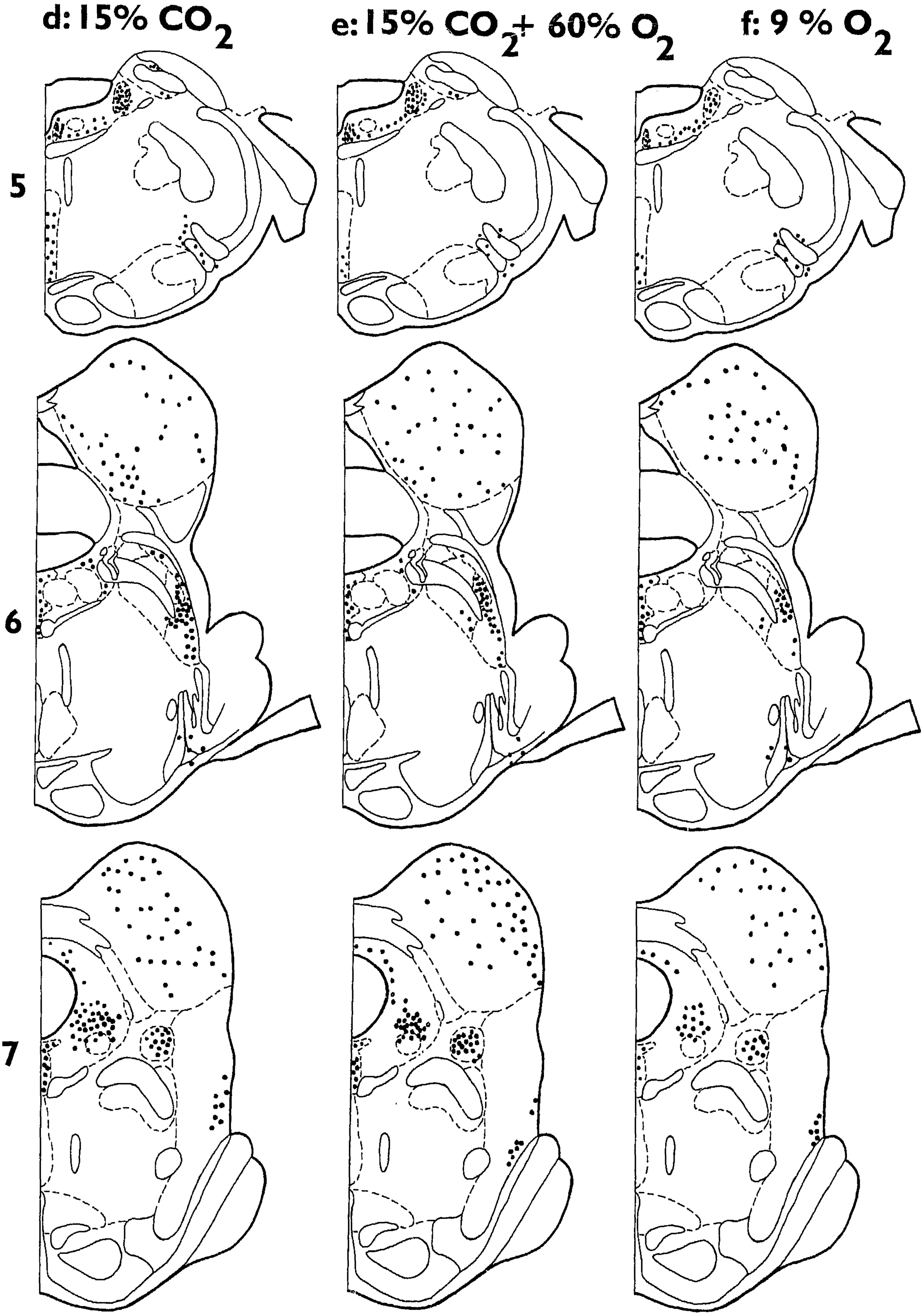


Figure 1 (Continued.)

of the medullary reticular nucleus (containing A1 noradrenergic cells) and the nucleus ambiguus (AMB); Level 2 (Fig. 1, row 2, approximately -13.76) was cut through the rostral border of area postrema and ventrally through the A1/C1 region; Level 3 (Fig. 1, row 3, approximately -11.90), through the rostral NTS, and ventrally through the retrofacial part of the lateral paragigantocellular nucleus (PGCl; see Andrezik et al., 1981) containing C1 adrenergic cells; Level 4 (Fig. 1, row 4, approximately -11.10) was cut through the juxtafacial level of PGCl, through the gigantocellular nucleus pars α (GiA) and the raphe magnus nucleus (RM); Level 5 (Fig. 1, row 5, approximately -9.80) contained the rostral pole of the locus coeruleus (LC) and the caudal ends of the parabrachial nucleus (PB) and the A5 noradrenergic cell group; Level 6 (Fig. 1, row 6, approx. -8.85), showing the PB complex including the Kölliker-Fuse nucleus (KF) and the rostral end of the A5 region; and Level 7 (Fig. 1, row 7, approximately -8.30), caudal mesencephalon with caudal periaqueductal gray (PAG) and the cuneiform nucleus (CUN).

Numbers of Fos-positive nuclei are given per hemisection only, because the brains of several animals were not cut perfectly perpendicular to the longitudinal axis. Fos immunoreactive nuclei were identified as highly condensed black spots, often with a fusiform shape and, at high magnification levels, frequently with a clearly discernible unstained nucleolus. TH-positive cells were easily recognizable by a yellow-orange staining of the cytoplasm. At the levels of interest, the number of TH-cells per hemisection were also counted, as well as those with a Fos-positive nucleus.

Statistics

Cell counts were performed and, for each individual section, repeated on the consecutive day. Differences between numbers of Fos-positive cells between two or more of the six stimulus paradigms were tested with ANOVA, using a fixed model. To defend against type I errors, a Bonferroni correction on the P value of 0.05 was made, depending on the number of comparisons within a given test.

The design of this study and the use of rats were approved by the Ethical Committee for Animal Experiments of Leiden University.

RESULTS

In Figure 1 the distribution of cells with Fos immunoreactivity is shown for seven rostrocaudal levels in six rats, each subjected to a different stimulus condition. At all levels shown (and also in all remaining animals), Fos-positive neurons were found bilaterally, generally with a symmetrical distribution.

Control animals

Generally, the medulla of control animals contained few cells with Fos, indicating a low level of basal expression of Fos in air-breathing animals. An exception was the presence of many labeled cells in the dorsal cochlear nucleus (see Fig. 1a4). In the dorsal medulla, scarce and inconsistent labeling was seen in the NTS (Fig. 1a1–a3) and sometimes also in the vestibular nuclei (not shown in Fig. 1). The ventral medulla of control animals sometimes contained a small number of immunoreactive cells in the raphe nuclei, in the LRN and PGCl including the A1 and C1 regions (Figs. 1a1–a4, 2), and occasionally in the ventral half of trigeminal sensory structures (not shown in Fig. 1).

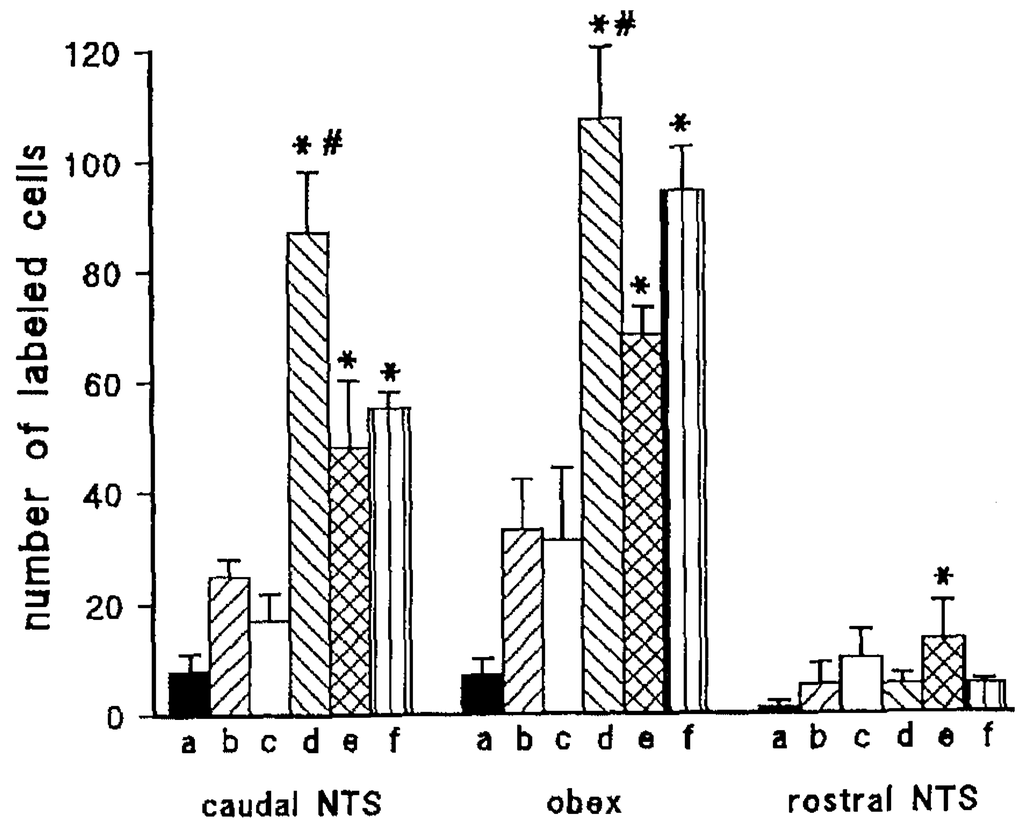


Fig. 2. Bar graph showing the mean number of labeled cells per 60-μm hemisection (± SD) in the nucleus tractus solitarius at three rostrocaudal levels after the six different challenge paradigms. The levels are approximately -14.36, -13.76, and -11.90 (Swanson, 1992). Asterisks at error bars denote significant differences with control (a), with P values <0.01. Pound signs denote significant difference from e (hyperoxic hypercapnia, $P = 0.002$). a: control, n = 9; b: 8% CO_2, n = 4; c: 10% CO_2, n = 5; d: 15% CO_2, n = 5; e: 15% CO_2 + 60% O_2, n = 4; f: 9% O_2, n = 7. For abbreviations, see list.

In the caudal pons, constitutive expression of Fos was encountered in dorsal midline structures (nuclei incerti (NI, Fig. 1a5) and dorsal raphe nucleus (DR, Fig. 1a6,a7) and pontine central gray (Fig. 1a6). Very few labeled cells were observed in PB and LC (Fig. 1a5,a6) and in (the vicinity of) the A5 cell-group. More rostrally, the number of Fos-positive cells in DR decreased (Figs. 1a7, 7).

In caudal midbrain, moderate levels of constitutive Fos expression were encountered in PAG, in CUN, in the inferior colliculi, and diffusely in the mesencephalic reticular nucleus, including the retrorubral area (Fig. 1a7).

Test animals

An example of the distribution of Fos-positive cells in the brainstem for all challenge paradigms is shown in Figure 1. In the bar graphs of Figures 2, 4, and 8 the mean numbers of labeled cells per hemisection in most areas of interest are summarized.

Dorsal medulla

After all challenge paradigms (except control), the NTS contained a large number of cells immunoreactive to Fos. Although exposure to 8 and 10% CO_2 already induced Fos expression (Figs. 1b1,c1, 3b,c), most labeled cells were present after 15% CO_2 (cf. Figs. 1d1, 3d); in the caudal NTS and at the level of the obex, addition of 60% O_2 to the inspiratory air reduced the number of immunoreactive cells (Figs. 1d1–2,e1–2, 2, 3d,e). After hypoxia, a smaller number of labeled cells appeared than after the normoxic exposure to 15% CO_2 (Figs. 1f1–3, 2, 3d,f). The area postrema of hypoxic rats contained labeled cells (Fig. 3f), but in hypercapnic animals this was only rarely observed (Figs. 1f2, 3d,e). In the caudal NTS of both the hypoxic and hypercapnic rats, the Fos-positive cells were mainly concen-

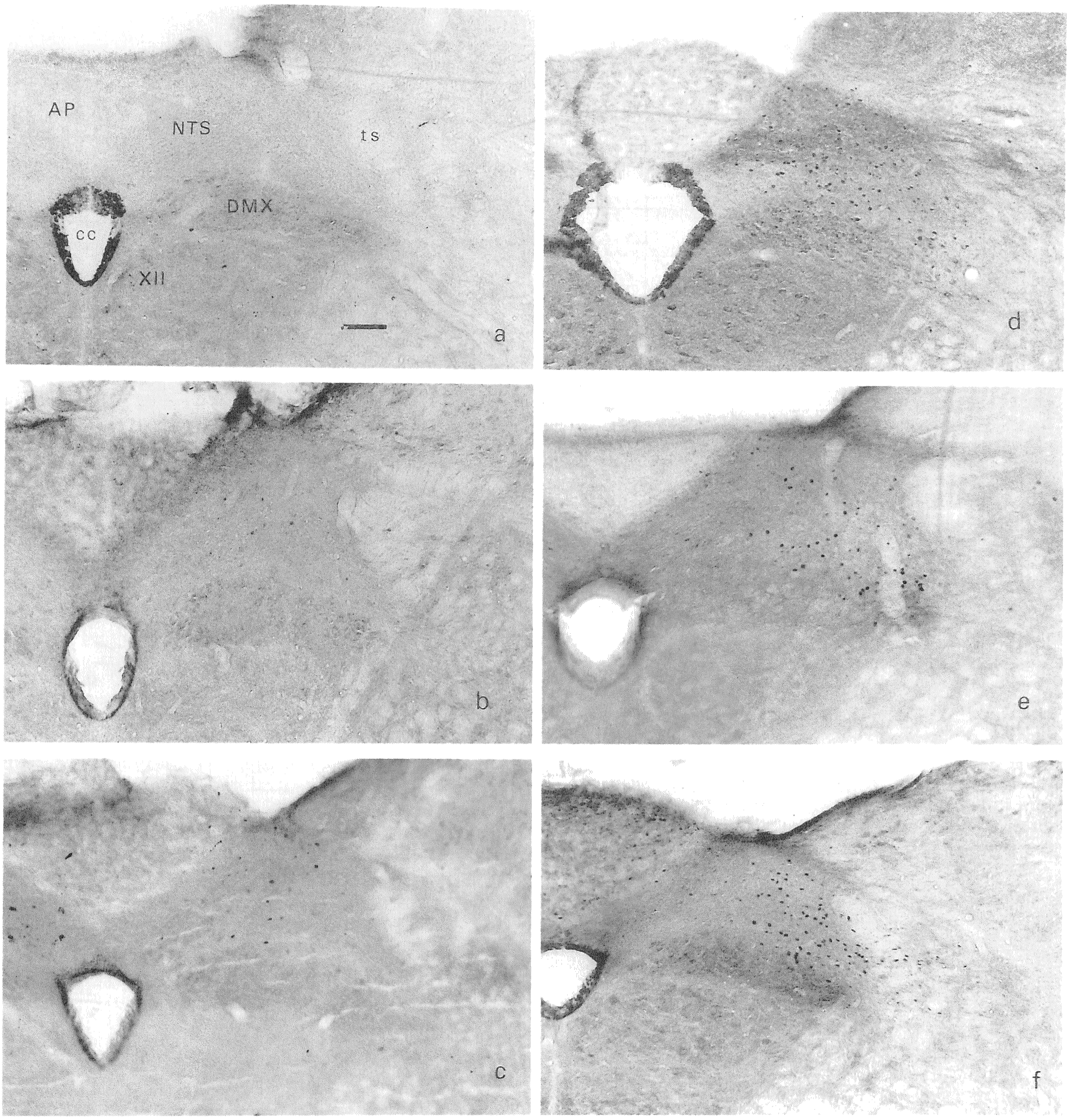
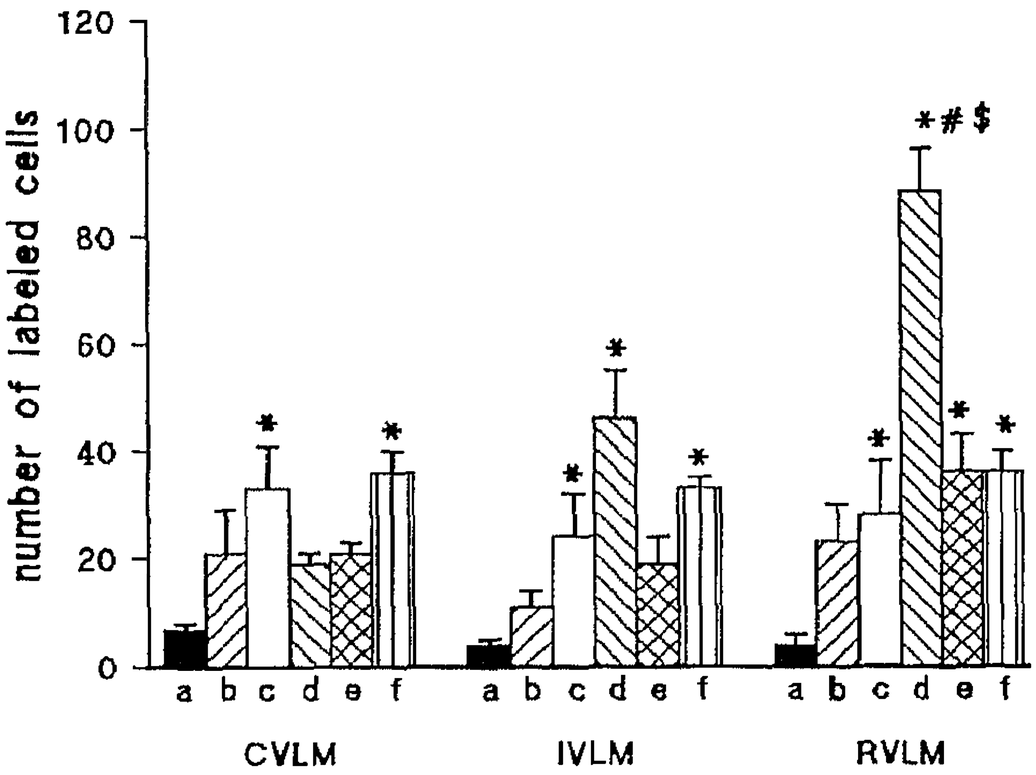


Fig. 3. Expression of Fos in the NTS (nucleus of the solitary tract) at the level of area postrema (approximately -14.36 = Fig.1, row 1). **a:** Air-breathing control; **b:** 8% CO_2 in air; **c:** 10% CO_2 in air; **d:** 15% CO_2 in air; **e:** 15% CO_2 + 60% O_2, balance N_2; **f:** 9% O_2, balance N_2. Scale bar = 100 µm in a (applies to a–f).

trated in the commissural, dorsomedial and medial subnuclei, although some labeling was also observed in the ventrolateral NTS. Rostral to the obex, the number of labeled cells in the medial interstitial and lateral subnuclei, decreased gradually, resulting in only a limited number of immunoreactive cells at levels rostral to the hypoglossal nuclei (Figs. 1, row 3, 2; see also Erickson and Millhorn, 1994).

Ventral medulla

Very few Fos-labeled cells were observed in the caudal parts of the raphe obscurus and pallidus nuclei (Fig. 1, rows 3 and 4). This was true for the control situation as well as for the test conditions. The only exception was after exposure to 15% CO_2, when a small number of immunoreactive cells was present within the raphe obscurus.



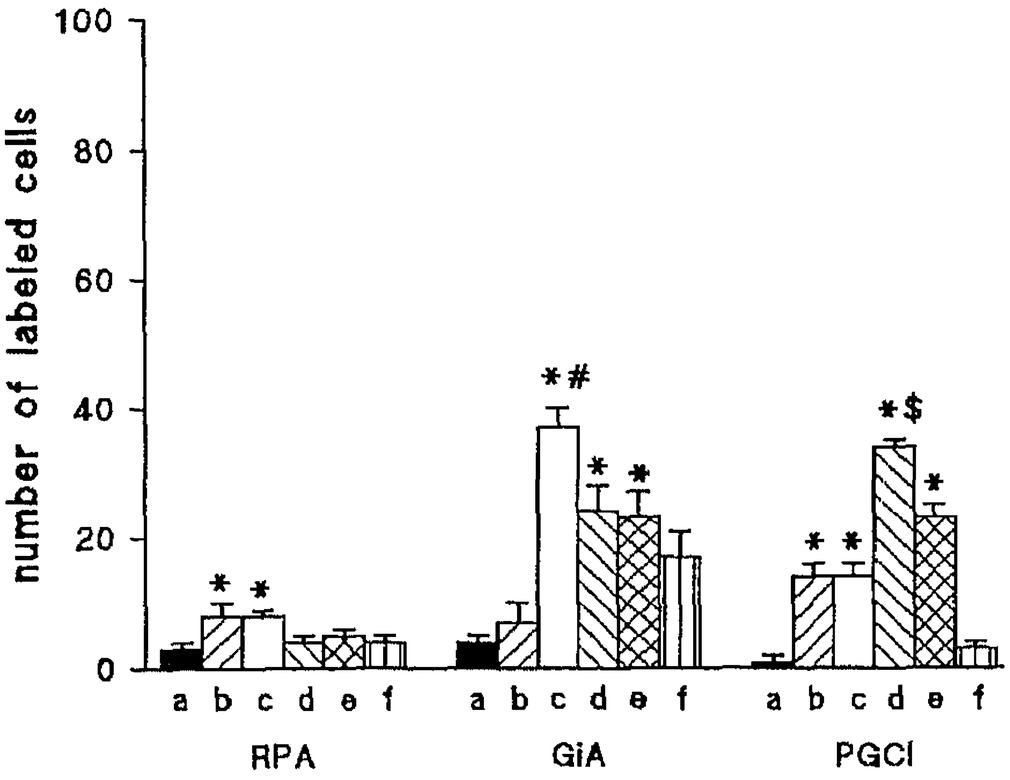


Fig. 4. Left: Bar graphs representing numbers of Fos-positive nuclei per 60-µm hemisection in ventral medulla. Left: cell counts at the same three rostrocaudal levels as in Figure 2 (cf. levels 1–3 in Fig. 1). Asterisks at error bars (SD) denote significant differences with air breathing controls (a), by using P values < 0.01 for the CVLM and IVLM (= intermediate [obex] level of the ventral lateral medulla), and < 0.007 for RVLM. Note the significant difference in labeling between 15% CO_2 (d) and 15% CO_2 + 60% O_2 (e) in RVLM, and that between 15% CO_2 (d) and hypoxia (f), denoted by pound signs and dollar signs, respectively. In IVLM, these differences were not tested. Right: Cell counts in the more rostrally located RPA, GiA and juxtafacial PGCl (approximately level 4 in Fig. 1). Asterisks denote significant differences from the air breathing controls (a), using P values < 0.01 for RPA, < 0.006 for GiA, and < 0.005 for PGCl. Pound signs denote significant difference from 9% O_2 (f; $P < 0.001$). Dollar sign denotes significant difference between 15% CO_2 (d) and hyperoxic hypercapnia (e; $P < 0.001$). a: control, n=9; b: 8% CO_2, n=4; c: 10% CO_2, n=5; d: 15% CO_2, n=5; e: 15% CO_2 + 60% O_2, n= 4; f: 9% O_2, n=7. For abbreviations, see list.

Caudal ventral lateral medulla. In caudal ventral lateral medulla (CVLM), many immunoreactive cells were found in and nearby the A1 noradrenergic cell group. In hypoxic and hypercapnic animals, most Fos-positive neurons were located between the nucleus (retro)ambiguus and the LRN (Fig. 1, row 1). Labeling was not confined here to the A1 noradrenergic cells, but was also present within and ventral to the LRN (Figs. 1, rows 1 and 2, 10a). The number of Fos-positive cells in the CVLM was not obviously different between the hypercapnic and hypoxic paradigms (Fig. 4). At the level of the obex, where the A1 and C1 cell-columns overlap, labeled cells were encountered in similar amounts (Fig. 1, row 2). At this level, the immunoreactive cells were located at 1.5–2.5 mm from the midline. Generally within the CVLM, the distance between the Fos-positive cells and the ventral surface ranged from less than 100 µm to approximately 750 µm. Occasionally, labeled cells were also observed on the ventral surface of the caudal medulla.

Rostral ventral medial medulla. In raphe pallidus, the effects of hypercapnia and hypoxia did not obviously differ: both resulted in limited Fos expression (Fig. 1, row 3). In GiA and RM, moderate numbers of labeled neurons were encountered. After hypoxia, labeled cells were somewhat less in number here than after hypercapnia (Figs. 1, rows 4 and 5, 4, 7c–e).

Rostral ventral lateral medulla. Within the rostral ventral lateral medulla (RVLM), including the C1 region, many Fos-positive cells were encountered, particularly in hypercapnic animals (Figs. 1, row 3, 5, 10d–f). Few labeled cells were seen dorsal to AMB. The largest number of immunoreactive cells was found after exposure to 15% CO_2. At the rostrocaudal level within the PGCl where cell counts were made [i.e., at the rostral half of the inferior olive complex, between levels 60 and 61 in the atlas of Swanson (1992)], addition of 60% O_2 to the hypercapnic inspiratory gas reduced the number of labeled cells (Figs. 4, 5b,c); hypoxic animals showed comparable numbers of Fos-positive cells as these hyperoxic rats (Figs. 4, 5c,d). Generally within the PGCl of hypercapnic animals, labeled cells were located at distances varying between 0 and 750 µm from the ventral surface (Figs. 1, row 3, 5, 10d–f).

From the rostral-most levels of the retrofacial PGCl to the caudal boundaries of the facial nucleus, including those levels where both the rostral end of AMBd and the caudal pole of the facial nucleus are visible, hypercapnic (10 and 15% CO_2) rats contained a very superficially located cell-column, which was absent in the hypoxic animals (Figs. 5b–e, 10d–f). Because this cell-column was visible in four to five (sometimes six) consecutive neighbouring sections, each containing several tens of immunoreactive cells, we estimate its rostral extent at approximately 500 µm. Most cells were located at depths between 0 and 100 µm. According to the double-staining results, this very superficial cell column consists of noncatecholaminergic cells and is located ventrally and ventrolaterally to the C1 adrenergic cells. This is illustrated in Figure 10d,e. The rostral boundary of this labeled cell-column was formed by superficial cells located within the very narrow region between the facial nucleus and the subarachnoidal space, mostly on the ventral medullary surface. Examples are also shown in Figure 6. Due to the fact that the pial surface was often somewhat compressed in our freezing microtome sections, it was not possible to reliably count the number of labeled cells lying on the surface ventral to the facial nucleus. As discussed below, we consider this superficial Fos-positive cell-column as part of the retrotrapezoid nucleus (RTN).

After the hypercapnic challenges, consistent bilateral labeling was found in the juxtafacial part of the PGCl

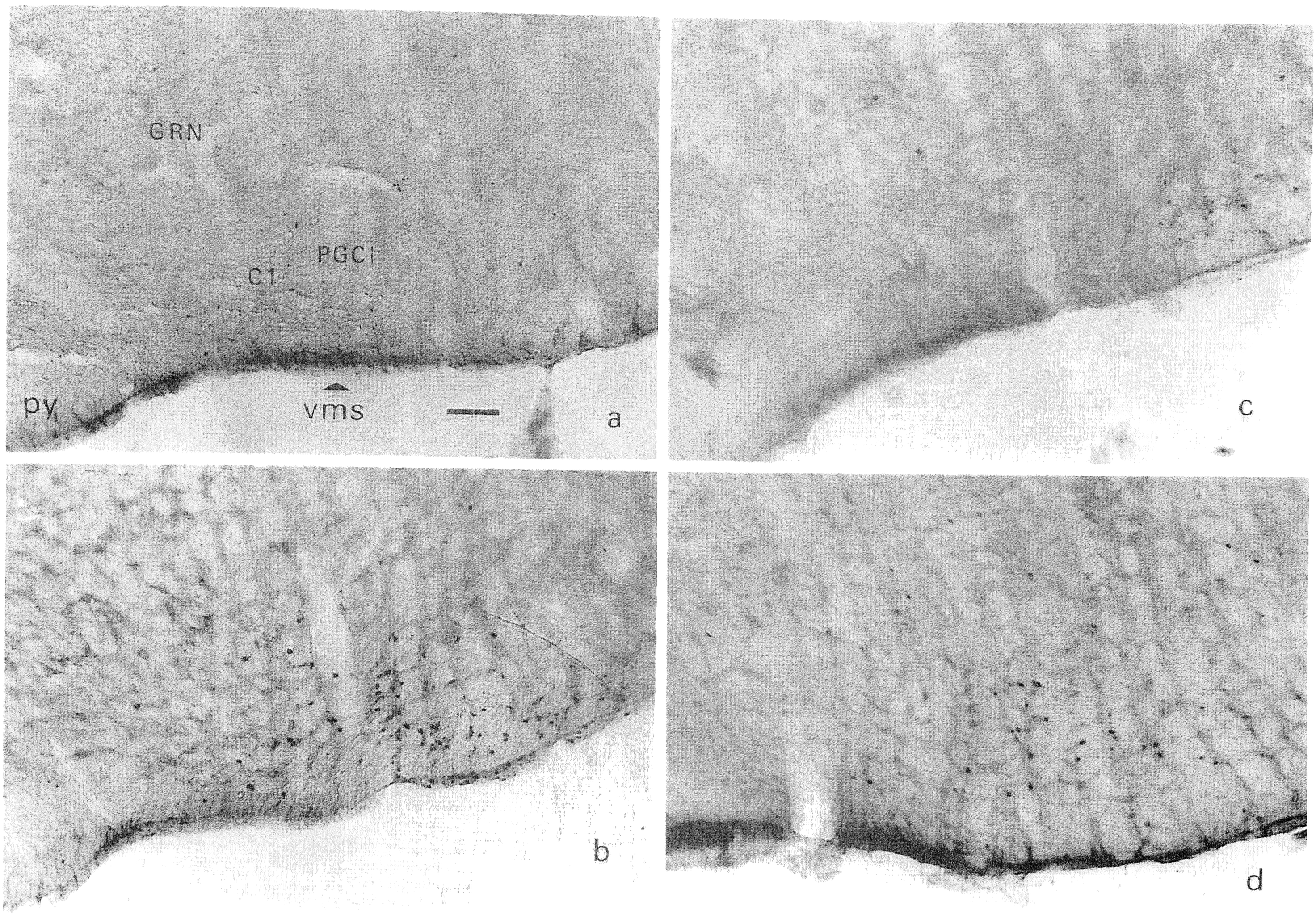


Fig. 5. Expression of Fos in the retrofacial PGCl (C1 region). **a:** control; **b:** 15% CO_2; **c:** 15% CO_2 + 60% O_2; **d:** 9% O_2. Exposure to 15% CO_2 (b) yields the largest number of labeled cells. Note superficially located cells in b and c. In hypoxia (d), few labeled cells are seen in superficial tissue. For abbreviations, see list. Scale bar = 100 μm in a (applies to a–d).

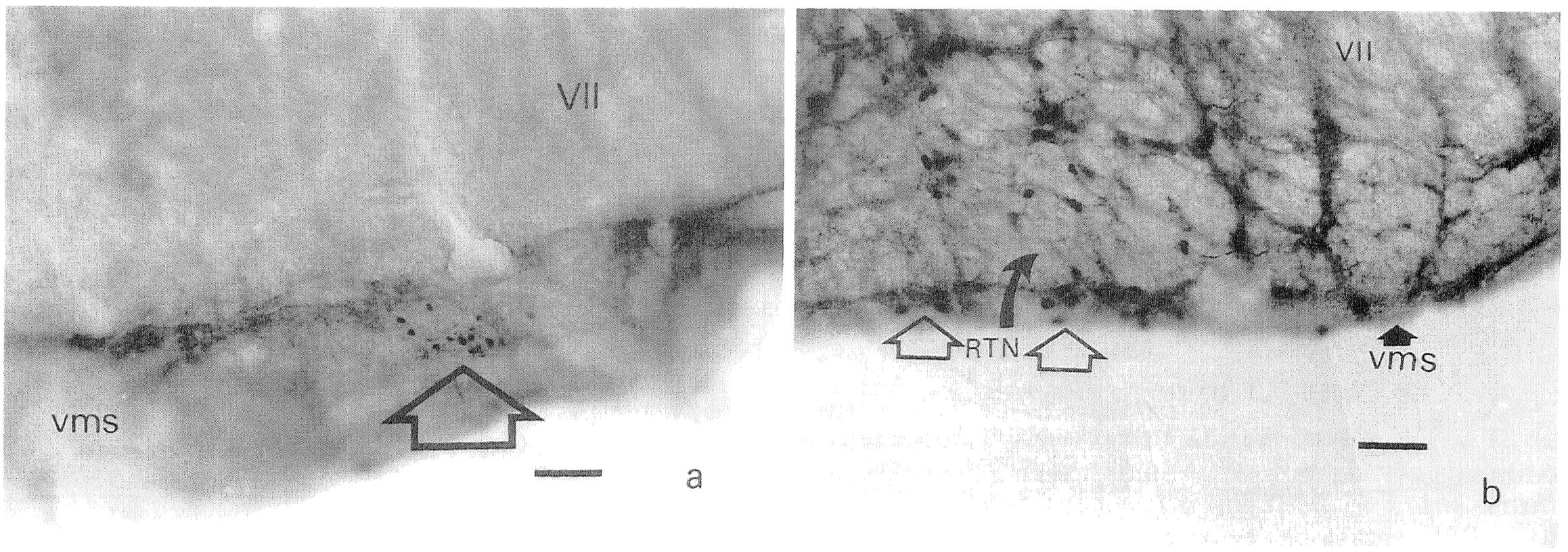
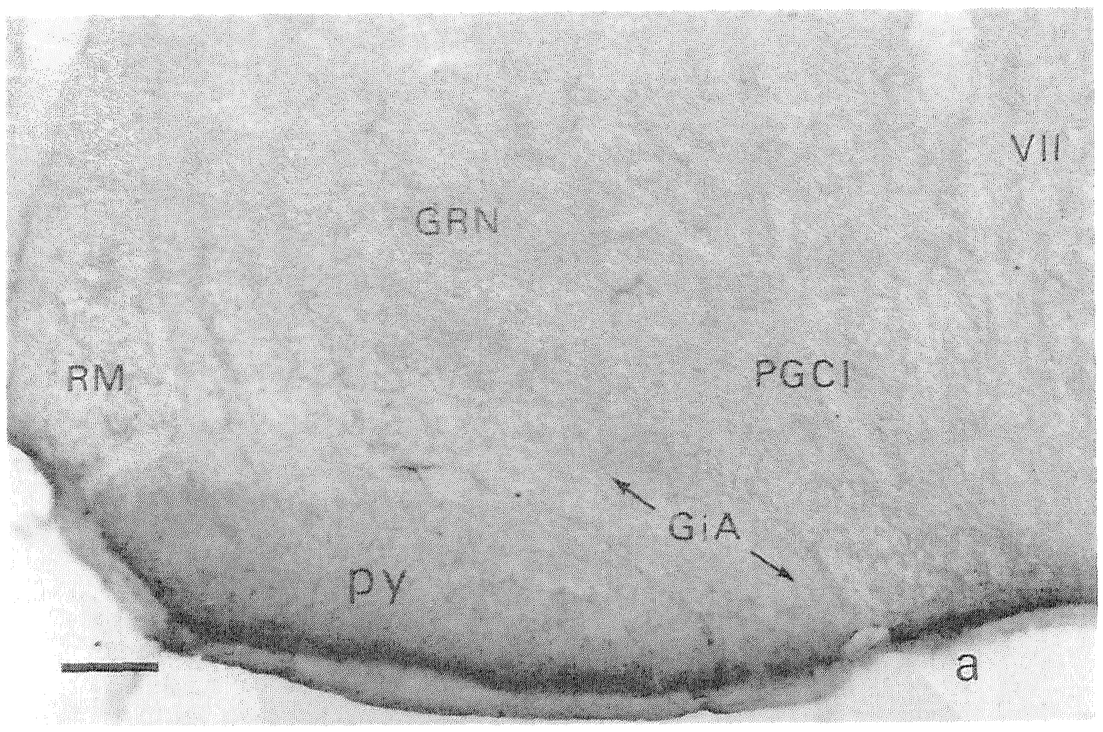
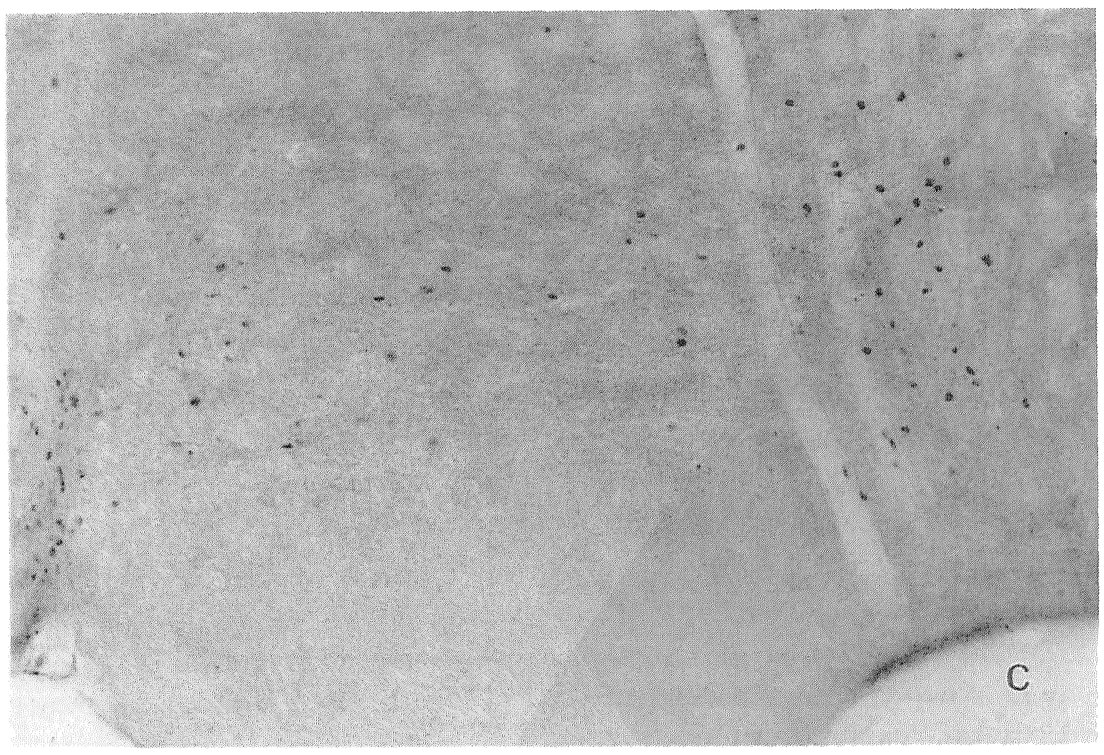
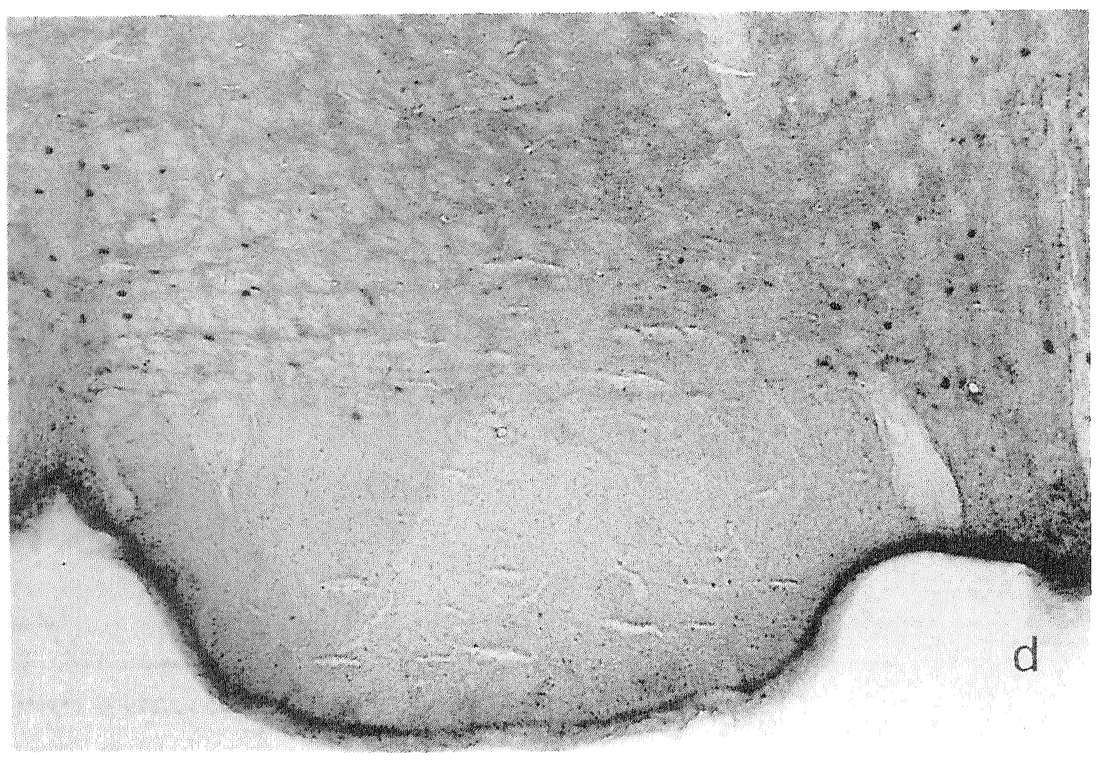


Fig. 6. **a,b:** Expression of Fos at the ventral medullary surface below the facial nucleus in two animals exposed to 15% CO_2. Open arrows show some obvious locations with labeled cells on the ventral medullary surface. Curved arrow indicates RTN (retrotrapezoid nucleus); filled arrow indicates vms (ventral medullary surface). Scale bars = 50 μm.

where the facial nucleus occupies its largest cross-sectional area (Fig. 1, row 4). These immunoreactive neurons were located medial to the facial nucleus, at depths between 100 and 750 μm. Rostrally, this labeling extended approximately to the caudal limits of the nuclei of the trapezoid bodies. Interestingly, this pattern of labeling within the juxtafacial PGCl was absent after exposure to the hypoxic gas (Figs. 1, row 4, 4, 7c–e).







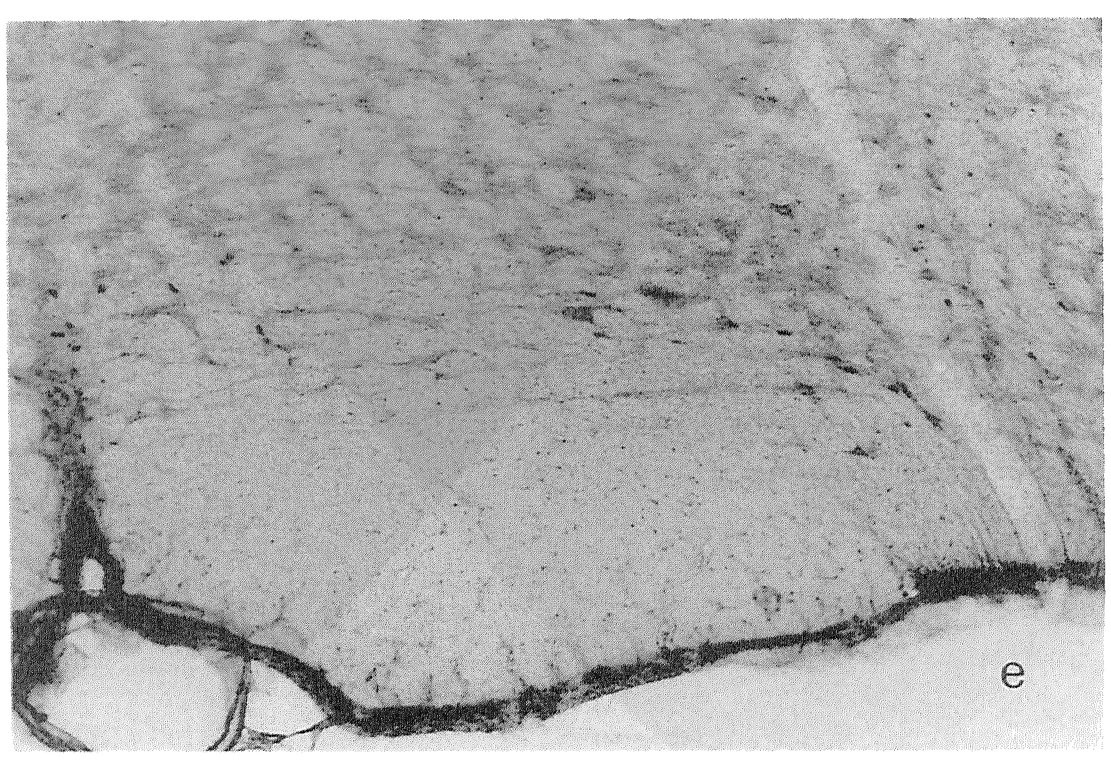


Fig. 7. Fos expression in RM (nucleus raphe magnus), GiA (gigantocellular nucleus pars α) and juxtafacial PGCl (paragigantocellular nucleus, lateral part). **a:** Air-breathing control; **b:** 8% CO_2; **c:** 15% CO_2; **d:** 15% CO_2 + 60% O_2; **e:** 9% O_2. Hypercapnia (b–d), induces expression of Fos in juxtafacial PGCl. For abbreviations, see list. Scale bar = 100 μm in a (applies to a–e).

Pontine level

Unlike Sato et al. (1992), we found many labeled neurons at levels rostral to the medulla oblongata. Many cells immunoreactive to Fos were observed in the parabrachial complex (PB), and, to a lesser extent, in locus coeruleus (LC) (Figs. 1, rows 5 and 6, 8). At both locations, the number of labeled cells was larger after exposure to hypercapnic than to hypoxic gas (Figs. 1, rows 5 and 6, 8, 9b,c,e,f, 10h,i). Within the LC, Fos-positive neurons were predominantly found in the rostral pole (Figs. 1, rows 5 and 6, 10h,i). Labeled cells were also encountered in the subcoeruleus region (subC), immediately ventral and ventrolateral to the LC, at levels rostral to the mesencephalic trigeminal nucleus. Labeling in PB was concentrated in the rostral part of the external lateral subnucleus, and in the Kölliker-Fuse nucleus ventrally and ventromedially to

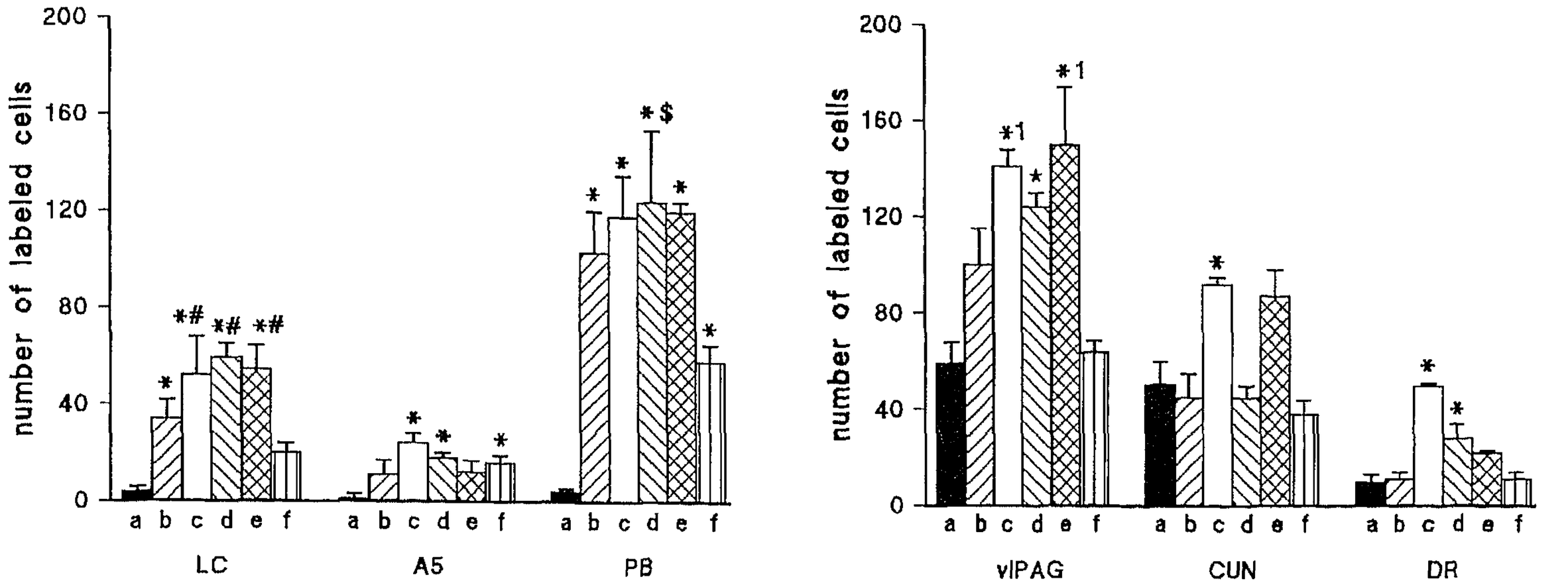


Fig. 8. Bar graphs presenting numbers (± SD) of Fos-positive cells per 60-µm hemisections from pontine levels 5 (LC and A5) and 6 (PB; left) and from caudal mesencephalon (level 7; right). Asterisks denote significant differences from air-breathing controls (a; $P < 0.01$ for A5, DR, and CUN, and < 0.006 for LC, PB and vlPAG, respectively). Pound signs in LC denote the number of Fos-positive cells is significantly larger after these hypercapnic exposures than after hypoxia (f; $P < 0.006$). The dollar sign in PB denotes a significantly larger number of labeled neurons after 15% CO_2 (d) compared with hypoxia (f; $P < 0.006$). The 1's in vlPAG denote the fact that 10% CO_2 (c) and 15% CO_2 + 60% O_2 (e) result in significantly more Fos-positive cells than hypoxia (f) does. a: control, n = 8; b: 8% CO_2, n = 4; c: 10% CO_2, n = 5; d: 15% CO_2, n = 5; e: 15% CO_2 + 60% O_2, n = 4; f: 9% O_2, n = 7. For abbreviations, see list.

the superior cerebellar peduncle (Figs. 1, row 6, 9b,c). Few labeled cells were present in the medial and in the central and dorsolateral subdivisions of PB.

Both after the hypercapnic and hypoxic challenges, Fos-positive neurons were found in the ventrolateral pons, in or adjacent to the A5 region (Fig. 10c; the A5 region is located between levels 5 and 6 in Fig. 1). The dorsal raphe (DR) at this pontine level showed an increased expression of Fos after 10 and 15% CO_2 (with or without hyperoxia). After exposure to 8% CO_2 and 9% O_2 labeling did not differ significantly from that in air-breathing controls.

Caudal mesencephalon

In the caudal ventrolateral PAG (vlPAG), with a moderate level of constitutive Fos expression, hypercapnia resulted in an increase in the number of Fos-cells while hypoxia had little influence (Figs. 1, row 7, 8, 9d–f). The labeled cells in vlPAG appeared as a characteristic cluster (Fig. 9d–f), with a rostrocaudal extension of approximately 360–480 µm (four to five neighbouring sections). In the cuneiform nucleus a significant increase in the number of immunoreactive cells was found after 10% CO_2 only (Figs. 1, row 7, 8, 9d–f). The dorsal raphe contained a significantly increased number of labeled cells after 10 and 15% CO_2 (Figs. 1, rows 6-8).

TH immunoreactive cells

The distribution of cells immunoreactive to TH was similar to that reported by Hökfelt et al. (1974), Kalia et al. (1985a,b), Erickson and Millhorn (1994), and, as far as the ventrolateral medulla concerns, Ellenberger et al. (1990). Examples of TH-containing cells in most regions of interest are shown in Figure 10. Briefly, in the dorsal medulla catecholaminergic cells were found in the A2 noradrenergic (Fig. 10a) and C2 adrenergic cell groups, and within the medial longitudinal fasciculus (C3 adrenergic cell group). In the ventral medulla, caudal to the obex, compactly arranged TH-containing neurons were found in the A1 noradrenergic cell group (Fig. 10b). Some of these cells contained long dendrites travelling to the ventral surface. More rostrally, from the level of area postrema to approximately the caudal end of the facial nucleus, scattered within the lateral tegmental field, a large amount (see Table 1) of TH-immunoreactive cells representing the C1 adrenergic cell group, was present (Fig. 10d–f; see also Kalia et al., 1985b, and Ellenberger et al., 1990). Many TH-containing dendrites contacting the ventrolateral medullary surface were observed here (see also Fig. 10d–f).

In the dorsolateral pons, a high concentration of TH-neurons was found within LC (A6 noradrenergic cell group, Fig. 10g–i). More rostrally, medial to the middle cerebellar pedunculi, TH-containing cells within KF, belonging to the A7 noradrenergic cell-group, were observed. In the ventrolateral pons the noradrenergic neurons of the A5 cell-group stained also for TH (Fig. 10c). In the midbrain, TH containing neurons were observed ventrolaterally from the cerebral aqueduct, representing catecholaminergic cells of the retrorubral A8 cell-group.

Neither the various hypercapnic, nor the hypoxic experimental paradigms affected the distribution and amounts of TH-containing cells (see Table 1).

Colocalisation of TH and Fos

Dorsal medulla. In caudal medulla (Fig. 1, row 1), the percentage of TH containing A2 neurons showing immunoreactivity to Fos was low, varying from 7% in the control situation to 23% after normoxic hypercapnia (15% CO_2). The data in Table 1 (see %Fos+TH) further show that the fraction of Fos-positive cells also containing TH was low. An example in a hypercapnic animal is shown in Figure 10a.

At the rostral boundaries of area postrema (Fig. 1, row 2), the percentage of double-labeled cells in the A2/C2 cell-group was larger. During normoxic hypercapnia, a maximum of 63% double-labeling was reached, which was reduced to only 28% during hyperoxia. In both conditions,

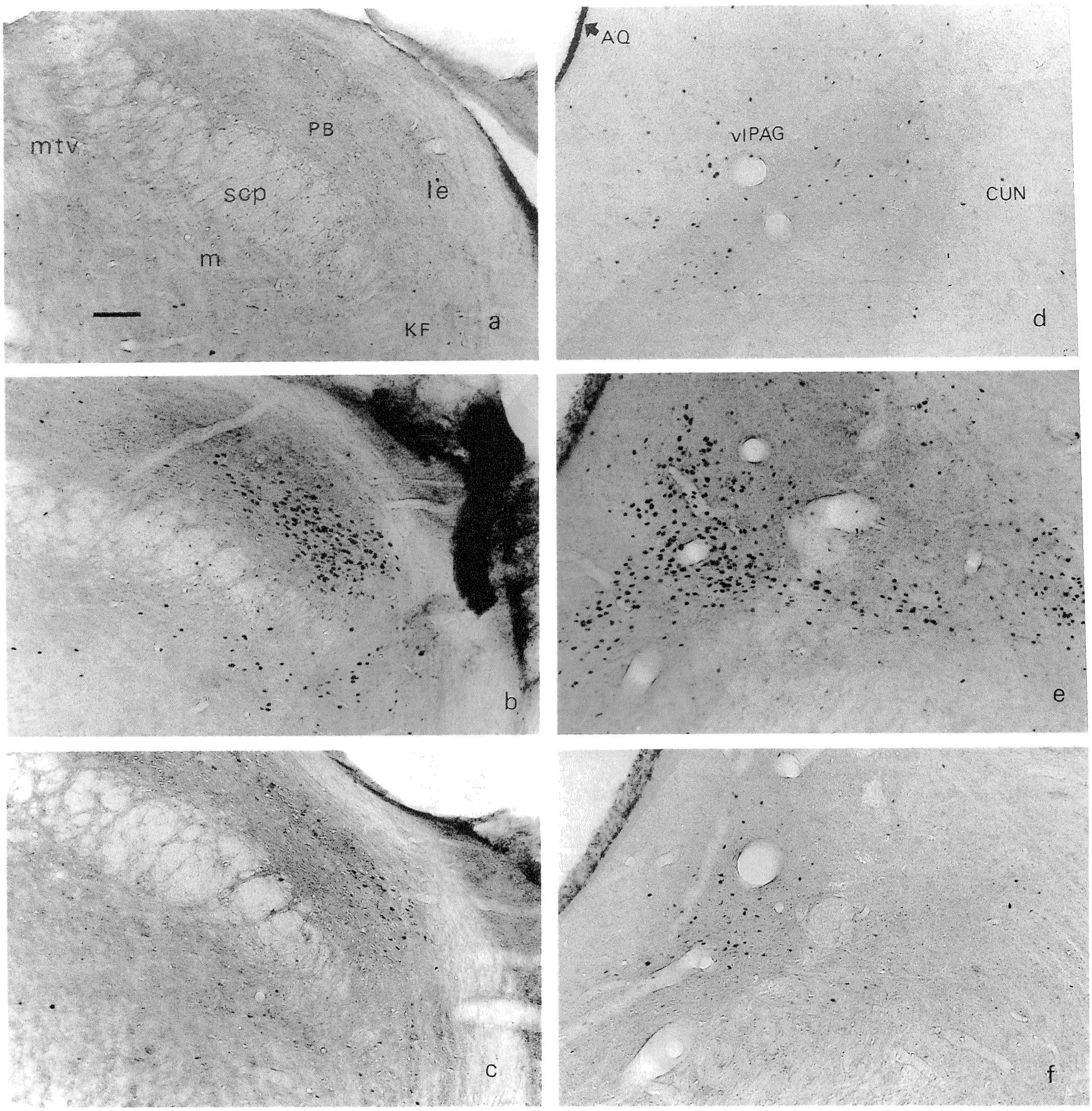


Fig. 9. Fos expression in PB (parabrachial nucleus) (a–c) and vlPAG (ventrolateral column of periaqueductal gray) and CUN (cuneiform nucleus) (d–f). **a,d:** air-breathing controls; **b,e:** 10% CO_2; **c,f:** 9%O_2. In PB, vlPAG and CUN, numbers of labeled cells are considerably larger after hypercapnia than after hypoxia. For abbreviations, see list. Scale bar = 100 μm in a (applies to a–f).

however, the number of noncatecholaminergic Fos-positive neurons was much larger than the number of labeled TH-containing cells.

Rostral to the obex (Fig. 1, row 3, cf. level 4 in Erickson and Millhorn, 1994), the number of cells labeled with Fos or TH was much lower, as was the number of double-labeled cells in the C2 adrenergic cell group. In the C3 cell group within the medial longitudinal fasciculus no Fos-cells were encountered, and also no double-labeling.

Altogether, from the data in Table 1 we conclude that in the dorsal medulla all stimulus paradigms (except the control situation) resulted in Fos expression in neurons which in majority were noncatecholaminergic. We remark, however, that cell counts were not limited to the boundaries of the A2 and C2 groups but extended to the entire NTS complex. The percentage of TH-containing NTS-cells activated by hypercapnia varied from low in the caudal NTS to somewhat more than 50% at the rostral end of area

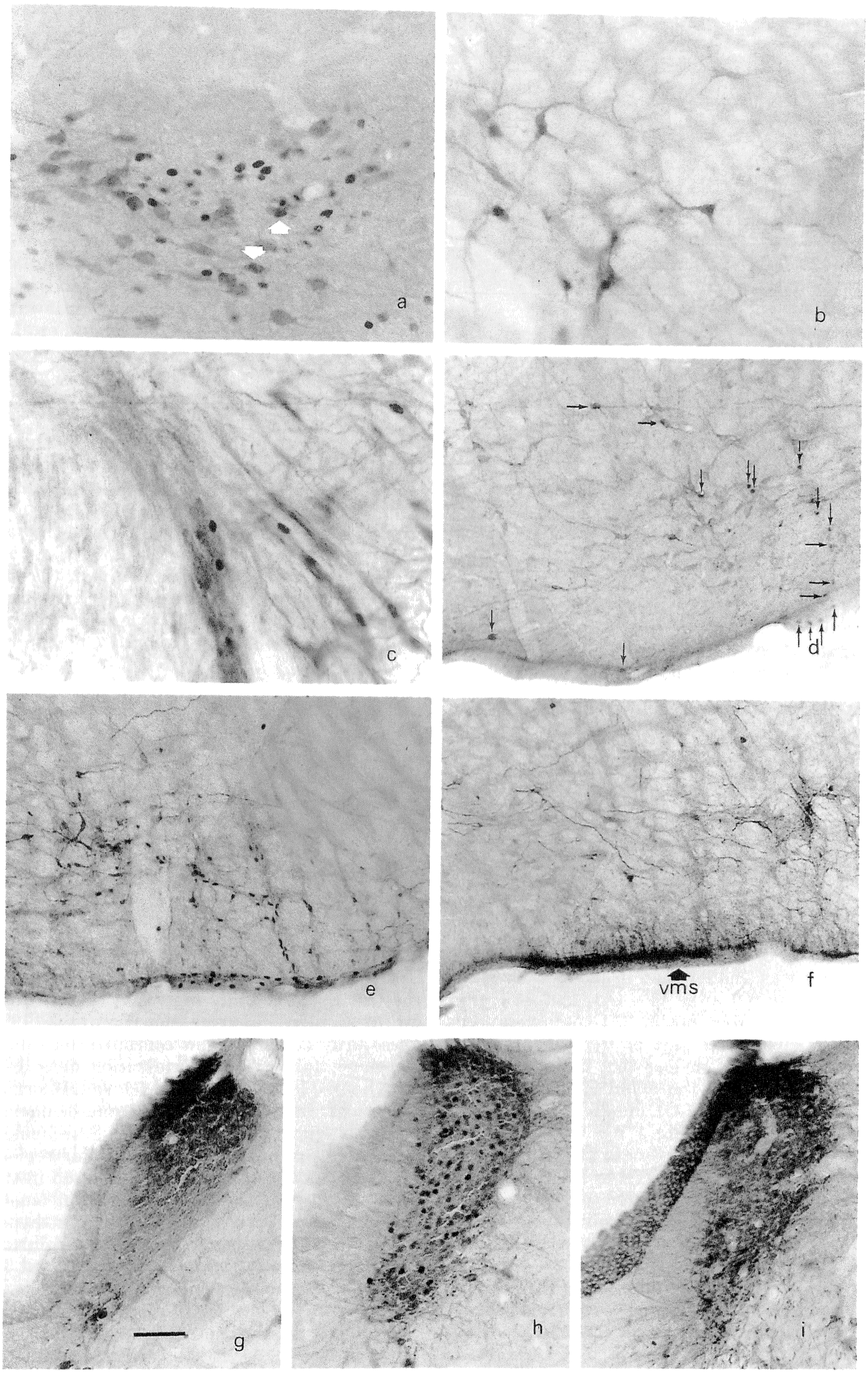


Fig. 10. Double immunostaining for Fos and TH (tyrosine hydroxylase). **a:** the A2 region from a hypercapnic (15% CO_2) rat. Two of the few double labeled cells are indicated by white arrows; **b:** the A1 region from a hypercapnic (15% CO_2) rat. Note large degree of colocalization of Fos and TH; **c:** the A5 region in a hypoxic rat, showing colocalization of Fos and TH. Note also some Fos-positive cells outside the A5 region; **d–f:** C1 region, from rats exposed to 10 and 15% CO_2 and to 9% O_2, respectively. Arrows in d (10% CO_2) show Fos-positive cells, few of which are catecholaminergic: colocalization of Fos and TH is less than in the A1 region. Note also that in the hypercapnic rats (d and e), superficial cells are noncatecholaminergic; **g–i:** LC (locus coeruleus) in, respectively, a control rat, a hypercapnic (15% CO_2) rat, and a hypoxic animal. Scale bar = 30 μm in a–c, 60 μm in d–i.

TABLE 1. Colocalization of Fos and TH[1]

	Level 1		Level 2		Level 3		
	A2	A1	A2/C2	A1/C1	C2	C1	A5
Control							
n = 3							
n TH	22	16	16	21	5	42	20
%TH + FOS	7	14	7	2	0	0	10
%FOS + TH	35	7	4	3	0	0	50
10% CO_2							
n = 3							
n TH	28	18	25	22	7	45	30
%TH + FOS	9	61	18	20	6	2	63
%FOS + TH	19	38	41	20	1	5	72
15% CO_2							
n = 4							
n TH	32	14	21	26	8	42	25
%TH + FOS	23	89	63	65	10	21	46
%FOS + TH	8	68	12	38	12	14	56
15% CO_2 + H							
n = 2							
n TH	30	11	22	30	8	58	25
%TH + FOS	9	75	28	30	5	7	62
%FOS + TH	8	46	10	75	2	14	70
9% O_2							
n = 3							
n TH	26	16	25	34	8	40	21
%TH + FOS	14	84	22	54	13	14	70
%FOS + TH	8	35	9	61	15	14	76

[1]n TH, number of TH cells per hemisection. Percentage sign TH + Fos, the percentage of TH-cells containing Fos; percentage sign Fos + TH, the percentage of Fos-positive neurons containing TH. Counting in the A5 region was done between levels 5 and 6 in Figure 1.

postrema after exposure to 15% CO_2 (in normoxia only). Hypoxia resulted in Fos expression in about one out of five to seven TH-cells.

Ventral medulla. A large percentage of TH-cells within the A1 noradrenergic cell-group was immunoreactive to the Fos antibody. During hypercapnia (15% CO_2) the maximum was 89%, and during hypoxia 84% (for example see Fig. 10b). At this medullary level (Fig. 1, row 1); however, the expression of Fos was not limited to the A1 noradrenergic cell-group alone because labeled cells were also present in the adjacent LRN and ambiguus complex (see above). At the level of area postrema (Fig. 1, row 2), where the A1 and C1 cell-groups overlap, the percentage of TH-cells with Fos was smaller than in the more caudally located A1 cell-group (65 after 15% CO_2 and 54% after hypoxia). Although in this region TH containing cells accounted for a substantial part of the appearance of Fos-immunoreactivity, it is obvious that the contribution of noncatecholaminergic cells was about equal (Table 1).

Near the rostral end of the C1 adrenergic cell group, posterior to the facial nucleus (Fig. 1, row 3), the degree of TH/Fos colocalization reduced further to 21 and 14%, after 15% CO_2 and 9% O_2, respectively (Table 1; Fig. 10e,f). In this region the majority of Fos-positive cells was noncatecholaminergic. Inspection at high magnification revealed that some activated TH-neurons had dendrites reaching the ventral medullary surface.

A5 cell group. In the A5 noradrenergic cell group, a relatively large degree of colocalization was found because both after hypercapnia and hypoxia (Fig. 10c) a large fraction (> two-thirds) of the cells with Fos contained TH (Table 1).

Locus coeruleus (A6 noradrenergic cell group). Colocalization of TH and Fos was frequently observed here. Quantitation, however, was not performed due to the very high density and immunoreactivity of TH-containing neurons (Fig. 10g–i).

A7 noradrenergic cell group. Generally, few TH cells in KF were Fos-positive. Most activated neurons were located dorsolateral to the noradrenergic cells.

DISCUSSION

General considerations

In this study we describe the distribution of Fos expression in the rat brainstem after hypercapnic and hypoxic challenges. Neither hypoxia nor hypercapnia are specific stimuli to the ventilatory control system because they give also rise to a variety of hormonal, cardiovascular, autonomic and stress-related adaptations. For example, both may result in an increase in sympathetic activity, in changes in blood pressure, in (non-uniform) changes in blood flow through peripheral organs, heart and brain, in activation of the hypothalamus-pituitary-adrenal axis, and in changes in body temperature and metabolism. However, because the respiratory control system selectively detects and corrects alterations in blood gas tensions, hypoxia and hypercapnia are the obvious means to stimulate lung ventilation. Furthermore, induction of c-fos requires strong stimulation (Dragunow and Faull, 1989), and in spontaneously breathing animals the hemodynamic changes caused by hypercapnia or hypoxia are much smaller than those needed to induce Fos expression in cardiovascular brainstem areas (c.f. Chan and Sawchenko, 1994; Murphy et al., 1994). In the conscious rat, inspiration of a gas mixture with 10% O_2 does not produce changes in mean arterial pressure from normoxic levels (Kregel, 1996). We did not measure arterial blood pressure in this study, and because we did not identify respiratory or respiratory related neurons, we cannot exclude that part of the labeled cells were activated by cardiovascular or stress-related sequelae of the manipulations. For this reason, we compared our data with those from Fos studies in which hemodynamic challenge paradigms were used (see below).

The described labeling patterns may not completely represent the consequences of hypercapnia and hypoxia: (1) The time pattern of Fos expression depends on the nature, strength and duration of a given stimulus. Thus the fact that generally high CO_2 concentrations are required to induce Fos expression (Sato et al., 1992), does not necessarily mean that low concentrations have no effect. Supplemental in situ hybridization data (cf. Chan and Sawchenko, 1994) would yield relevant information in this respect. (2) Inhibited neurons cannot be envisaged by the Fos technique (see also Chan and Sawchenko, 1994). (3) Lack of Fos expression in a particular region cannot be taken as evidence that it is not involved in the response, and therefore some false negative outcomes cannot be excluded. Respiratory motoneurons in the hypoglossal and ambiguus nuclei, although clearly excited during hypercapnia, do not appear to up-regulate *c-fos*—at least on the time scale studied.

Comparing different Fos studies can be difficult. First, anesthesia can influence the expression of *c-fos*, and different anesthetics have distinct effects (Dragunow and Faull, 1989; Krukoff, 1994; Takayama et al., 1994; Dampney et al., 1995). Second, the use of different antibodies and the application of various histochemical and experimental procedures (e.g., strength and duration of stimuli) may sometimes render a realistic comparison difficult. For example, Erickson and Millhorn (1994), in part of their

study, performed CSN stimulation for only 10 minutes, while in other studies by using hypoxic, pharmacological, or hemodynamic challenges much longer stimulation periods were applied.

Fos immunohistochemistry may be a valuable tool in the localization of central chemoreceptors, but for the time being a few reservations can be made in this respect. Synaptic activation is known to increase the expression of Fos, but as yet it is unknown if this also can be achieved by a direct action of CO_2. In addition, it is unknown if there exists a causal relationship between the hypercapnic increase in ventilation and Fos expression in the brainstem. Data from studies using anti-sense Fos mRNA are not yet available. Increased synthesis of Fos is functionally related to long-term cellular adaptation (Morgan and Curran, 1986, 1989, 1991), but, conceivably, such adaptations could be quite relevant if, during hypercapnia, they would occur in chemosensitive cells. The *acute* response to hypercapnia does not appear to require de novo protein, or RNA synthesis, because it is not affected by anisomycine and actinomycine D, respectively (Bayliss et al., 1990).

Dorsal medulla

The location of Fos-positive neurons within the caudal NTS after the hypoxic and hypercapnic challenges corresponds with known projection sites of carotid body chemoafferents in the commissural and medial subnuclei of the rat NTS, preferentially at and posterior to the obex (Housley et al., 1987; Finley and Katz, 1992; Chitravanashi and Sapru, 1995). Sensory information on changes in $Paco_2$ and Pao_2 travels within the same glossopharyngeal nerve fibres. Thus the finding that hypoxia resulted in labeling at the same locations as hypercapnia indicates that the immunoreactive cells within the NTS represent second order neurons involved in the processing of chemoafferent information from the carotid bodies. The decreased labeling in the caudal and intermediate NTS during hyperoxic hypercapnia compared with normoxic hypercapnia (see Fig. 2) may thus be due to the known interaction between CO_2 and O_2 at the level of the carotid bodies (Lahiri and DeLaney, 1975; Fitzgerald and Deghani, 1982). In the caudal NTS, more labeled cells appeared after inhalation of 15% CO_2 than after exposure to 9% O_2. In conscious rats, the latter challenge leads to a hypocapnic arterial PCO_2 of about 3 Kpa (Pepelko and Dixon, 1975; Kregel, 1996), which may have resulted in a lower carotid body activity than during the exposure to 15% CO_2, thus explaining the smaller number of labeled cells in the caudal NTS.

The NTS contains cells that are directly sensitive to CO_2 (Dean et al., 1990; Coates et al., 1993), and these may represent part of the observed labeled neurons in hypercapnic rats. Most Fos-positive cells in the caudal NTS during hypoxia and hypercapnia (except 15% CO_2) were noncatecholaminergic (see also Erickson and Millhorn, 1994). Isovolumic hypotension yields a similar pattern (Chan and Sawchenko, 1994), which could be the result of the known chemoreceptor activation by hypotension. However, in the A2/C2 region the percentage of TH-containing neurons labeled with Fos was larger than in the caudal NTS (Table 1, level 2), and this may indicate reflex activation of the NTS-hypothalamic-pituitary axis, particularly during exposure to 15% CO_2 (references see Chan and Sawchenko, 1994).

Ventral medulla

Caudal ventral lateral medulla. The site of labeled cells in CVLM of hypoxic and hypercapnic animals corresponds partly with the location of the A1 noradrenergic cell group (Kalia et al., 1985a). Our results show that a large fraction of A1 noradrenergic neurons stained for Fos. However, a substantial part of the Fos-positive cells in CVLM was noncatecholaminergic (see Table 1). This finding differs from that after systemic hypotension, when activation in this medullary region is mainly limited to A1 cells (Chan and Sawchenko, 1994; Li and Dampney, 1994). After hypertension in conscious rabbits and rats, however, few catecholaminergic A1 neurons are activated (Chan and Sawchenko, 1994; Li and Dampney, 1994). So apparently, the labeling patterns after hypo- and hypertension differ from those after both hypoxia and hypercapnia, when more ventrally located cells (for example ventrolaterally to the LRN), and neurons close to the medullary surface appear to have increased levels of Fos. During hypoxia and hypercapnia, CVLM neurons could be activated by (also labeled) caudal NTS neurons, which are known to project to A1-cells and to noncatecholaminergic neurons in this medullary region (Sawchenko and Swanson, 1981; Ross et al., 1985). The methods used in this study do not have the resolution to discriminate between the following possible sequelae of activation by hypoxia and hypercapnia of noradrenergic A1 and neighbouring noncatecholaminergic neurons: (1) activation of vasopressin-synthesizing neurons in the hypothalamus to which A1-cells project (e.g., Sawchenko and Swanson, 1982; Cunningham and Sawchenko, 1988); (2) reflex inhibition of sympathetic output from RVLM (C1) neurons to which noncatecholaminergic neurons of the CVLM project (e.g., Day et al., 1983); (3) activation (and labeling) of adjacent retroambigual cells of the caudal ventral respiratory group (CVRG), with which A1 neurons in fact are intermingled (Ellenberger et al., 1990; Sun et al., 1994). Part of the labeled noncatecholaminergic cells in CVLM may be cells projecting to the phrenic motor nucleus or to respiratory neurons in the PGCl or KF nuclei (Loewy et al., 1981; McKellar and Loewy, 1982).

The area ventral to the A1 neurons is analogous with part of the caudal chemosensitive area in the cat (see Schlaefke, 1981). The labeled cells in the hypercapnic rats were less superficially located here than previously reported in rats exposed to 13% CO_2 (Sato et al., 1992). No labeling in the CVLM was reported in awake and anesthetized cats, respectively, exposed to 5 and 10% CO_2 (Larnicol et al., 1994; Teppema et al., 1994). Because hypercapnia and hypoxia yielded comparable numbers of Fos-positive cells at the same locations within the CVLM, expression of the *c-fos* gene may not be due to direct actions of CO_2 or O_2.

Rostral ventral medial medulla. The rostroventromedial medulla (RVMM) is a region containing many serotonergic cells with a sympathoexcitatory and/or inhibitory role independent from that of the RVLM (references see Chalmers and Pilowsky, 1991; Zagon, 1995). In this study we found that hypercapnia and, to a lesser degree hypoxia, induced Fos expression in raphe magnus (RM) and the GiA. The distribution of labeled cells in GiA was very similar to that of the serotonin containing cells within the B3 serotonergic cell group (Loewy et al., 1981; Erickson and Millhorn, 1994). In raphe pallidus (RPA) we observed

a moderate number of labeled cells after exposure to 8 and 10% CO_2 only (Fig. 4).

In other studies it has been shown that medullary raphe structures are involved in respiratory control. For example, medullary raphe nuclei have (reciprocal) connections with the RVLM (Zagon, 1995) and other cardiorespiratory brainstem regions (Connelly et al., 1989; Smith et al., 1989; Holtman et al., 1990). Projections to phrenic motoneurons do also exist (Dobbins and Feldman, 1994), and in the cat these may be serotonergic (Pilowsky et al., 1990). Connections between GiA, at levels where we found Fos-positive neurons, and phrenic motoneurons have also been reported (Dobbins and Feldman, 1994; Onai et al., 1987). Electrophysiological data in the cat showed that medullary raphe nuclei contain neurons with respiratory modulated activity, some of which show respiratory-phase-dependent synchrony with respiratory neurons in the ventrolateral medulla (Hosogai et al., 1993; Lindsey et al., 1994). Bernard et al. (1996) showed that microinjection of acetazolamide in the RVMM at a rostrocaudal level where we found Fos-positive neurons, can cause considerable increases in phrenic nerve activity. The existence of neurons within RM and GiA with direct sensitivity to CO_2 was reported by Richerson (1995).

Rostral ventral lateral medulla. That part of the rostral ventral medulla where we found many cells immunoreactive to Fos, particularly after hypercapnia, has been termed rostroventrolateral medulla (RVLM, e.g., Ross et al., 1984, 1985), subretrofacial area (e.g., McAllen, 1986; Dampney, 1994), retrofacial PGCl (Andrezik et al., 1981), rostral ventrolateral nucleus (Saper, 1994) or the C1 region (e.g., Hökfelt et al., 1974; for further references see Chalmers and Pilowsky, 1991). The ventral border of this area is analogous to the intermediate chemosensitive area in the cat (Schlaefke, 1981). The RVLM is crucial in the control of cardiorespiratory, autonomic, and arousal functions and in the mediation of pain (e.g., Schlaefke, 1981; Millhorn and Eldridge, 1986; Dampney and Goodchild, 1987; Guyenet et al., 1990; Chalmers and Pilowsky, 1991; Reis et al., 1994; Van Bockstaele and Aston-Jones, 1995).

The location of labeled cells in the RVLM of hypercapnic animals was not limited to very superficial regions (<200 μm depth) as reported by Sato et al., (1992), but extended to depths of about 750 μm. The number of (noncatecholaminergic) immunoreactive cells was reduced by about 50% when the hypercapnic inspirate (15% CO_2) was made hyperoxic (cf. Fig. 5b,c), suggesting involvement of the peripheral chemoreceptors. The intermediate, subretrofacial, chemosensitive area not only contains central chemosensitive units, but is also involved in the integration of afferent input from the peripheral chemoreceptors (Millhorn and Eldridge, 1986). Neurons within the commissural and medial subnuclei of the NTS send projections to bulbospinal sympatho-excitatory C1 adrenergic neurons, as well as to neurons containing different neurotransmitters (Ross et al., 1985; Hancock, 1988), suggesting that the peripheral chemoreceptors may be able to influence the activity of noncatecholaminergic neurons in the RVLM. This may also be indicated by our observations because neurons expressing Fos during normoxic but not hyperoxic hypercapnia (15% CO_2) did not contain TH. A dense projection from the caudal, commissural, NTS to the PGCl, especially to regions ventral to the NA has been shown by Van Bockstaele et al. (1993).

Hypoxia and hypercapnia are known to activate RVLM reticulospinal vasomotor neurons, an effect partly mediated via the peripheral chemoreceptors (Hanna et al., 1981; Lioy, 1989; Reis et al., 1994). Direct effects of both hypoxia and hypercapnia on RVLM neurons, however, have also been described (Lioy, 1989; Sun and Reis 1993; Reis et al., 1994), and these may be responsible for part of the Fos-expression in this study [although the hypoxic stimulus used in our study was much less intense than that applied by Sun and Reis (1993)]. In the C1 region, we found only a low percentage of double-labeling of TH-containing neurons (see Table 1). In this respect our results are different from those obtained after depressor challenges, when a large fraction of bulbospinal C1 adrenergic cells shows expression of Fos (McAllen et al., 1992; Chan and Sawchenko, 1994; Li and Dampney, 1994; Murphy et al., 1994). During hypertension, the number of Fos-positive neurons in the C1 region appears to be low and mainly confined to noncatecholaminergic cells located preferentially dorsal to the C1 cell group (Chan and Sawchenko, 1994; Li and Dampney, 1994), compared with a more ventral location of labeled cells in the present study. It thus follows that the labeling pattern that we found in the present study differs from those obtained both after hypo- and hypertension.

A consistent observation was the presence in hypercapnic but not hypoxic rats of Fos-positive cells in very superficial layers of the rostral end of the RVLM (Figs. 5, 6, 10), located within the intermediate chemosensitive area. This cell column, with a location corresponding to that of the retrotrapezoid nucleus (Pearce et al., 1989), extended over a length of approximately 0.5 mm, from levels at the rostral pole of the inferior olive [where the cells lie ventral to the C1 adrenergic neurons (see Fig. 10e)] to those including the caudal limits of the facial nucleus. Labeled cells, actually lying on the ventral medullary surface were frequently observed here, and may represent central chemoreceptors. Further rostrally, the region between the facial nucleus and the subarachnoidal space (which in the rat is very narrow) also contained labeled cells, albeit less in number. This contrasts with hypercapnic cats, where Fos expression in the RVLM was preferentially located ventrally and ventromedially to the facial nucleus (Teppema et al., 1994). In ruminants, the relative dimensions of the facial nuclei are larger than in cats, leaving less space available for the more ventrally located RTN. This could have resulted in a caudal displacement of neurons with functions similar to those of the RTN in the cat, thus explaining a preferential location of activated neurons in the area just posterior to the facial nucleus. Apart from neurons with respiratory modulated activity (Pearce et al., 1989; Connelly et al., 1990), the RTN may also contain central respiratory CO_2 chemoreceptors (Nattie and Li, 1990; Coates et al., 1993).

Juxtafacial PGCl. Rostrally, the PGCl is bordered laterally by the facial nucleus, and this region is called juxtafacial PGCl (Andrezik et al., 1981). The occurrence of labeled cells after hypercapnia but not hypoxia in the juxtafacial PGCl, may indicate local presence of CO_2 chemoreceptors. However, because the area has widespread connections, for example with NTS, PB and KF (Van Bockstaele et al., 1993; Van Bockstaele and Aston-Jones, 1995), (part of) these labeled cells may be synaptically activated. Thus the decrease in labeling in hyperoxic compared with normoxic hypercapnia (Fig. 4) could be due

to less activation of caudal NTS neurons (see Fig. 2). Alternatively, the lack of Fos-positive neurons in the juxtafacial PGCl (and their smaller number in the retrofacial part) during hypoxia could be related to its function in arousal and alertness functions (Van Bockstaele and Aston-Jones, 1995). In awake rats, exposure to 10% O_2 results in a Pa_{O_2} of approximately 30 mm Hg (Pepelko and Dixon, 1975; Kregel, 1996), and this may cause a decrease in their level of arousal. We could not observe behavioral differences in this respect between hypercapnic and hypoxic animals, because in both groups drowsiness was a regular observation (the animals were studied in their light period). We cannot exclude, however, that part of the difference in labeling between hypercapnic and hypoxic rats was due to a lower arousal state in the latter.

Pontine level

Locus coeruleus. The locus coeruleus is important in the modulation of sensory processing by the brain and is activated by a variety of stressful somatic and autonomic stimuli (Aston-Jones et al., 1991; Van Bockstaele and Aston-Jones, 1995). Activity in LC neurons is highest during wakefulness, particularly in conditions requiring increased alertness (Aston-Jones et al., 1984). Hypercapnic animals showed the largest number of labeled neurons in LC and SubC (Fig. 8). Fos-positive cells were also observed after hypoxia, but their number did not significantly differ from control (Figs. 8, 10g–i). These results are reminiscent of the effects of hypercapnia and hypoxia on the in vivo firing behaviour of LC neurons in the rat: Elam et al. (1981) showed that the stimulating effect of hypercapnia was independent from the integrity of the peripheral chemoreceptors, whereas the rather inconsistent effects of hypoxia were reversed by carotid body denervation. Our finding that the number of Fos-positive neurons in the LC did not differ between normoxic and hyperoxic hypercapnia may indicate that these neurons were indeed activated independently from the peripheral chemoreceptors.

The difference in labeling in LC between hypoxia and hypercapnia could be explained by a possible presence of cells that are directly sensitive to CO_2 (Coates et al., 1993). Alternatively, it could be due to a possible decrease in the level of consciousness: the LC receives a major—noncatecholaminergic—input from both the retro- and juxtafacial PGCl, and these pathways are involved in the role of the LC in the control of arousal functions and in the processing of cardiorespiratory and sensory information (Van Bockstaele and Aston-Jones, 1995). The low level of Fos expression in LC in hypoxia may be due to lack of activation by (also unlabeled) juxtafacial PGCl neurons. During hypercapnia, the converse could be the case, explaining the obviously increased labeling in LC neurons.

Some data indicate that LC neurons are specifically involved in respiratory functions. For example, injection of Pseudo Rabies Virus into the phrenic motoneurone pool results in third-order labeling—possibly via respiratory premotoneurons in the ventrolateral medulla—in the LC and SubC, at locations corresponding to that of the Fos-positive neurons in the present study (Dobbins and Feldman, 1994). Second, the processing of afferent input by the NTS may be modulated by LC neurons (Pérez and Ruiz, 1995). Recently, it was reported that noradrenergic LC neurons send axons to spinal motoneurons, where they may participate in the control of limb and respiratory movements (Fung et al., 1994).

A5 region. Our data show a high degree of TH/Fos colocalization in the A5 noradrenergic cell group during hypoxia (see also Erickson and Millhorn, 1994) and hypercapnia. A5 neurons receive baroreceptor input: they are inhibited by a rise, and excited by a fall in arterial pressure, and project to the intermediolateral cell column (Byrum and Gyuenet, 1987; Huangfu et al., 1991). A5 cells are involved in the sympathetic chemoreflex (Koshiya and Guyenet, 1994; Guyenet and Koshiya, 1995), so one may indeed expect their activation during hypoxia and hypercapnia. Some A5 neurons have a respiratory rhythm (Byrum et al., 1984). Ellenberger and Feldman (1990) showed anatomical connections with respiratory (pre)motoneurons in the RVLM, and injection of Pseudo Rabies Virus into the phrenic motoneurone pool results in third-order labeling in the A5 region (Dobbins and Feldman, 1994). The tonic inhibitory influence on respiratory activity by the caudal ventrolateral pons in an in vitro neonatal rat brainstem-spinal cord preparation is probably located in the A5 region, because local injections of α-adrenergic antagonists increase respiratory frequency (Hilaire et al., 1989). Micro-injections of muscimol in the vicinity of A5 neurons, or local electrical lesions in vagotomized rats results in apneustic breathing (Jodkowski et al., 1994). Afferents from CVLM and KF (for references see Saper, 1994) may be involved in the activation of A5 neurons during hypercapnia and hypoxia.

Parabrachial complex. The pontine respiratory group of the parabrachial complex is known as the pneumotaxic centre (Feldman, 1986). The PB complex is also an important cardiovascular and visceral relay station, utilizing an extensive network of connections with autonomic brainstem and forebrain nuclei (Fulwiler and Saper, 1984; Moga et al., 1990; Saper, 1994).

In this study we found that Fos-positive neurons were mainly concentrated in (the outer part of) the external lateral subnuclei (Pble), and in the Kölliker-Fuse nuclei. Recently, Chamberlin and Saper (1994) showed that microstimulation with glutamate of neurons in the Pble resulted in hyperpneic responses in rat. In the cat a respiratory role of the lateral PB complex was also demonstrated (Hirano et al. 1994). Neurons within the Pble receive afferent input from the (dorso)medial and commissural parts of the NTS (Herbert et al., 1990), and by this pathway they may have been activated during hypercapnia and to a lesser extent during hypoxia. The Pble does not project to the RVLM (Fulwiler and Saper, 1984; Herbert et al., 1990; Chamberlin and Saper, 1992) and has no mono- or multisynaptic connections with phrenic motoneurons (Dobbins and Feldman, 1994). On the other hand, it sends afferents to the central nucleus of the amygdala and the median preoptic nucleus (Fulwiler and Saper, 1984; Moga et al., 1990). In both regions, we found increased expression of Fos after exposure of rats to hypercapnia and hypoxia (Teppema et al., 1996). So, as already has been suggested for the cardiovascular (pressure) and respiratory responses elicited by microstimulation (Chamberlin and Saper, 1992, 1994), the Pble may play a role in the physiological adaptation to hypercapnia via ascending connections.

We have no additional means, other than the available anatomical descriptions (Chamberlin and Saper, 1992), to decide whether or not the lateral crescent area, which in contrast to the Pble has connections with the RVLM (Herbert et al.,1990; Chamberlin and Saper, 1992), contained labeled cells. However, in some animals exposed to

hypoxia most labeled cells were encountered in the outer zone of the Pble, or, alternatively, in the lateral crescent zone (for an example see Fig. 9b).

Labeling in KF was confined to its rostral third. Similar to the NTS and RVLM, the KF nucleus contained the largest number of Fos-positive neurons after exposure to hypercapnia. Because this part of KF has many reciprocal connections with the RVLM, and receives afferent input from the ventrolateral NTS, both regions (which were also activated) may have contributed to the activation of KF neurons. Stimulation independently from these regions cannot be excluded, because normoxic and hyperoxic hypercapnia resulted in similar number of labeled cells in KF (cf. Figs. 2, 4, 8).

Caudal mesencephalon

Periaqueductal gray. The PAG can be divided into four longitudinal columns, each with a characteristic cytoarchitecture and separate anatomical connections (Bandler et al., 1991; Carrive, 1993). We found that hypercapnia but not hypoxia induced Fos expression in the caudal ventrolateral PAG. The dorsomedial, dorsolateral and lateral columns showed a moderate basal Fos expression which was not influenced by one of the test paradigms. The ventrolateral column receives afferents from the medial nucleus of the NTS (Herbert and Saper, 1992), the ventrolateral medulla and from several higher centres, for example the central nucleus of the amygdala and the bed nucleus of the stria terminalis (for references see Saper, 1994). These regions may have contributed to the observed expression of Fos. Stimulation of the ventrolateral PAG column results in a decrease in sympathetic activity (Bandler et al., 1991; Carrive 1993). Generally, the PAG is an important structure for respiratory adjustments to different arousal states and for the coordination of respiration and vocalization (Harper et al., 1991; Ni et al., 1990; Davis and Zhang, 1991). The vlPAG is involved in some aspects of the defense reaction (Carrive, 1993), and the present data indicate that it may contain a subpopulation of neurons involved in the respiratory and/or autonomic adaptation to hypercapnic stress.

Cuneiform nucleus and dorsal raphe. Additional studies are necessary to investigate whether hypercapnia consistently induces Fos expression in CUN. Our observation of Fos expression in dorsal raphe during hypercapnia (10 and 15% CO_2) could be compatible with the known modulatory action of DR neurons on hypothalamic cells involved in autonomic control (for references see Saper, 1994).

CONCLUSION

The neuronal pathways involved in the ventilatory adaptation to hypercapnia and hypoxia are only partly known. Some regions showing Fos-positive neurons during hypercapnia, for example RTN, GiA, juxtafacial PGCl, LC, and vlPAG are not located within the classic brainstem respiratory centres, and may contain central CO_2 chemoreceptors. According to our data, the central processing of hypoxia and hypercapnia may at least partly occur at distinct locations. Indications for direct effects of hypoxia could not be obtained, possibly because we applied much less intense hypoxic stimuli than Reis and co-workers (Sun and Reis 1993; Reis et al., 1994).

A major advantage of Fos mapping studies is that activated cells can be located individually. By offering selective stimuli one is able to gain more insight into the function of separate subnuclei of complex structures such as NTS and PB. For example, it is interesting that the medial subnucleus of the PB complex, which is part of the pneumotaxic centre contained few Fos-positive neurons after hypercapnia, in contrast to the external lateral subnucleus which recently has been associated with the control of breathing (see Chamberlin and Saper, 1994). Whatever its cause (no activation, or, alternatively, an undetected, different time course of expression?), the lack of Fos-positive cells Pbm means that during hypercapnia it behaves differently from Pble.

The approach used in the present study did not enable us to discriminate between stress, cardiovascular, or respiratory sequelae of hypoxia and hypercapnia. At some locations, however, we showed a labeling pattern distinct from that after hemodynamic challenges. Selective microstimulation, application of microlesions, use of tracers and treatment with antisense-Fos MRNA would further clarify the role of different subnuclei in the neural control of breathing.

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